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Alessia Verani Borgucci

Role of monocytes in the pathogenesis of HIV-1 infection

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

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Abstract

Peripheral blood monocytes and tissue macrophages play critical roles in both the natural history and the pathogenesis of HIV-1 infection. This thesis mainly focused on the study of a potential defensive role of macrophages in HIV-1 infection.

HIV-1 expression in monocyte-derived macrophages (MDM) infected *in vitro* is known to be inhibited by lypopolysaccharide (LPS), the main component of the bacterial cell wall. However, the mechanisms are not completely understood. We show herein that LPS protects primary macrophages from infection by CCR5-dependent HIV-1 isolates. Inhibition was largely mediated by the release of the C-C chemokines RANTES, MIP-1α and MIP-1β. Indeed, (a) addition of LPS to MDM resulted in the vigorous production of RANTES, MIP-1α and MIP-1β; (b) high levels of CCR5, the C-C chemokine receptor, were expressed by MDM at the time of infection; (c) antibody-mediated depletion of RANTES, MIP-1α and MIP-1β neutralized the ability of LPS-conditioned supernatants to inhibit HIV-1 replication in MDM; (d) a combination of recombinant RANTES, MIP-1α and MIP-1β blocked HIV-1 infection as effectively as LPS itself.

CXCR4 mediates the entry of syncytia-inducing strains, both primary and T cell line-adapted. The ability of SI HIV-1 isolates to infect primary human macrophages has been disputed. Here, we report that CXCR4 expression on human MDM was variable but consistently significant. Primary CXCR4-dependent HIV-1 strains infected MDM productively and were specifically blocked by SDF-1. By contrast MDM supported the entry but not the replication of CXCR4-dependent TCLA HIV strains. Thus, monocyte/macrophages

support the entry and replication not only of CCR5-dependent, but also of CXCR4-dependent primary HIV-1 isolates.

Because CXCR4 is a functional coreceptor for HIV-1 infection of human macrophages, we investigated whether LPS also affects the replication of CXCR4-dependent HIV-1 isolates. Our results show that LPS inhibits the replication of X4 HIV-1 isolates in MDM through the release of novel soluble suppressive factor(s) that are as of yet uncharacterized.

to my father and my mother

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ABBREVIATIONS

ADE	antibody-dependent enhancement
Ag	antigen
AIDS	acquired immunodeficiency syndrome
bp	base pair
CCR	CC chemokine receptor
CTL	cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
ECL	extracellular loop
ELISA	enzyme-linked immunosorbent assay
Env	envelope
ER	endoplasmic reticulum
EU	exposed uninfected
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
HTLV	human T-lymphotropic virus
ID50	50% infectious dose
IFN	interferon
IN	integrase
LAM	lipoarabinomannan
LPS	lipopolysaccharide
LTNP	long-term non-progressor
LTR	long terminal repeat
M-tropic	macrophage-tropic
MDC	macrophage-derived chemokine
MDM	monocyte-derived macrophages
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP-1α/β	macrophage inflammatory protein 1α/β
NGF	Nerve growth factor
NLS	nuclear localization signal

NSI	non syncytia inducing
PBMC	peripheral blood mononuclear cells
OI	opportunistic infection
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
RANTES	regulated upon activation, normal T cell expressed and secreted
RT	reverse transcriptase
R5	CCR5-dependent HIV-1
R5X4	dualtropic, CCR5/ CXCR4-dependent HIV-1
SDF-1	stromal cell derived factor-1
SI	syncytia inducing
SIV	simian immunodeficiency virus
TCLA	T cell line-adapted
TLR	toll-like receptor
X4	CXCR4-dependent HIV-1

This thesis has been written by myself and has not been used in any previous application degree. All results were obtained by myself, with the following exceptions: the competitive PCR amplification to determine the levels of CCR5 mRNA on macrophages (section 4.1.5) was performed by Manola Comar (ICGEB, Trieste) Semiquantitative PCR analysis of HIV-1 viral DNA load, (section 4.1.5 and 4.2.4) was performed by Eleonora Tresoldi (Immunobiology of HIV Unit, DIBIT), and Elena Pesenti, respectively. Gabriella Scarlatti (Immunobiology of HIV Unit, DIBIT) characterized all primary HIV-1 isolates for coreceptor usage.

The results described in section 4.1 and 4.2 have been published in the following articles:

1) Verani, A., Scarlatti, G., Comar, M., Tresoldi, E., Polo, S., Giacca, M., Lusso, P., Siccardi, A.G., and D. Vercelli, C-C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells. J Exp Med, 1997. **185**(5): p. 805-16.

2) Verani, A., Pesenti, E., Polo, S., Tresoldi, E., Scarlatti, G., Lusso, P., Siccardi, A.G., and D Vercelli, CXCR4 is a functional coreceptor for infection of human macrophages by CXCR4-dependent primary HIV-1 isolates. J Immunol, 1998. **161**(5): p. 2084-8.

The results described in section 4.3 are the subject of a manuscript in preparation.

1 Introduction

1.1 HIV-1

In the early 1980s, reports from the United States described an increased incidence of *Pneumocystis carinii* pneumonia and an aggressive form of Kaposi's sarcoma in previously healthy young homosexual men [77, 78, 155]. Because a common finding in these patients was a depletion of $CD4^+$ T lymphocytes, the symptoms were ascribed to immunosuppression. The new disease was termed "acquired immunodeficiency syndrome" (AIDS) [79]. Within 2 years, the aetiological agent was isolated by three independent laboratories and referred to as lymphadenopathy-associated virus (LAV), human T-lymphotropic virus III (HTLV-III), and AIDS-related retrovirus (ARV) [22, 134, 221]. This novel agent was subsequently renamed human immunodeficiency virus type 1 (HIV-1) [68]. HIV-1 was shown to be transmitted via sexual contact and blood products, as well as from mother to child. The number of reported AIDS cases increased dramatically in the following years and HIV-1-infected individuals were also diagnosed in Europe and Central Africa [66, 80], in addition to the United States. HIV-1 infection has since then spread to all continents, affecting people regardless of race, sex or age.

Despite the fact that AIDS was first described in the United States and later in Europe, HIV-1 is believed to have originated from Africa [263]. In 1986, an antigenic variant of HIV-1, called HIV-2, was isolated from patients with AIDS-like illnesses living in Guinea-Bissau and other neighbouring countries of West Africa [65]. HIV-2 appears to be less transmissible and pathogenic than HIV-1 [390]. Characterization of antibodies in stored sera showed that reactivity

against HIV-1 and HIV-2 could be detected in Africa as early as 1959 [263] and 1966 [190], respectively. Further evidence for the African origin of HIV-1 came through the identification of the simian immunodeficiency viruses (SIVs), which are phylogenetically related to HIV-1 and HIV-2. Monkeys infected in the wild - such as Sooty mangabeys, Sykes, and African green monkeys - all harbour their own SIV variant (SIVsm, SIVsyk, or SIVagm) [130, 188, 258, 368]. In addition a lentivirus designed SIVcpz has been found in chimpanzees [282]. SIV infection does not appear to cause immunodeficiency in natural hosts [140]. By contrast, SIV isolates from several Asian macaque species held in captivity in primate research facilities in the United States, including SIV from rhesus macaques (SIVmac) and nemestrina macaques (SIVmnz), cause a fatal AIDS-like disease [86, 219, 258].

When viral genomes were compared at the nucleotide level, it was reported that HIV-1 and HIV-2 strains generally exhibit only around 40% homology [161], while HIV-2 is more closely related to certain SIV variants, such as SIVsm [129]. Cross-species transmission of SIV from monkeys to humans has been proposed as the origin of both HIV-1 and HIV-2 [184, 282]. A virus (SIVsm) that is genomically undistinguishable from HIV-2 was found in substantial numbers of wild-living sooty mangabeys whose natural habitat coincides with the epicentre of the HIV-2 epidemic [55, 137, 173]. Regardless of the origin of HIV-1, a recent paper documented that two chimpanzee subspecies found in central and eastern Africa, *P.t. troglodytes* and *P.t. schweinfurthii*, harbour SIVcpz. The viruses isolated from the two subspecies form two highly divergent phylogenetic lineages. Interestingly all the HIV-1 strains known to infect humans, including HIV-1 groups M, N and O, are phylogenetically closely related to SIVcpz strains that infect *P.t. troglodytes*, a

primate whose natural range coincides precisely with areas of HIV-1 group M, N, and O endemicity. Chimpanzees are commonly hunted and butchered, especially in west equatorial Africa, and as a consequence represent a ready source for zoonotic transmission of SIVcpz to man [135].

1.1.1 *In vivo* HIV-1 infection

The major target for HIV-1 infection are cells that express the CD4 surface antigen. CD4 normally functions as a ligand for major histocompatibility complex (MHC) class II molecules during antigen presentation, and is expressed on T-helper cells and cells of the mononuclear phagocytic lineage including monocytes, macrophages, dendritic cells, and brain microglial cells. HIV infection of CD4-negative cells has also been reported [50, 62, 203, 210, 223, 305], but it is usually much less efficient than infection of $CD4^+$ cells. Sexual transmission of HIV-1 occurs through mucosal surfaces and is the major route of infection worldwide. Analysis of genital biopsies from HIV-infected women has demonstrated that subepithelial macrophages represent the main target cells for HIV infection in the female genital tract [158]. Whether intact genital epithelium is a barrier to, or an active participant in HIV transmission has not been elucidated so far [38, 180].

HIV disease may be divided into three phases: a) primary infection b) clinical latency and c) clinically apparent disease culminating in AIDS. During primary infection, the virus has been found to rapidly travel from the port of entry (the bloodstream and/or mucosal surfaces) to regional and then distal lymph nodes, where it is trapped on the surface of follicular dendritic cells. Lymphoid tissue represents a major reservoir for the virus in infected individuals, and viral replication occurs in this tissue throughout the course of

infection [278]. Upon primary infection with HIV-1, patients may develop an influenza-like or acute mononucleosis-like illness. The period shortly after primary infection is marked by high numbers of virions and virus-infected cells in the blood accompanied by transient CD4 lymphocytopenia. Following the appearance of anti-HIV-1 antibodies (seroconversion) the viral load commonly decreases, probably as a result of increasing cytotoxic T lymphocyte (CTL) activity. The subsequent clinically asymptomatic period can last for several years. Subsequently, the viral load increases again and the number of $CD4^+$ T cells slowly declines. Gradual quantitative and qualitative loss of effector cell functions causes an impairment of both cellular and humoral immune responses. The resulting generalized immunodeficiency ultimately leads to the development of opportunistic infections, neurological disorders, or neoplasias such as Kaposi's sarcoma or B-cell lymphomas.

The median time from primary HIV-1 infection to the development of AIDS is approximately 10 years; however, the course of the disease can range from rapidly progressive to long-term asymptomatic (>10 years). The conditions underlying the long-term asymptomatic state have been the focus of intense investigation. The strongest prognostic marker identified to date is viral load [243, 244]. Indeed, long-term non-progressors (LTNPs) are characterized by low levels of virus in cells and plasma, vigorous immune responses, both humoral and cellular, a stable CD4 count, and no signs of symptoms of disease for many years [220, 335]. A few LTNPs have been found to harbour viruses defective in their accessory genes, most commonly *nef* [41]. It has been proposed that these virus variants may have a reduced replicative capacity in vivo. By contrast, rapid progressors show a rapid increase in viral

load and a parallel decline in CD4 counts, and consequently progress to disease faster than other infected individuals [110, 191, 200].

The level of HIV replication in patients is strongly influenced by both viral and host factors. Among the latter, the endogenous cytokines that control the homeostasis of the immune system, as well as inflammatory and immune-mediated reactions, play a very important role [117, 293]. In HIV-infected individuals there is increased secretion of the proinflammatory cytokines TNF-α, IL-6 and IL-1β in PBMCs and higher expression of these cytokines, together with interferon (IFN)-γ, in lymphoid tissues. Elevated levels of TNF-α and IL-6 are also found in plasma and cerebrospinal fluid [42, 212]. Overproduction of these cytokines could contribute to AIDS pathogenesis; in fact, IL-6 and TNF-α have been reported to induce HIV-1 expression in infected cells by acting at the transcriptional or post-transcriptional level [102, 292] and enhanced serum levels of IL-6 have been associated with the subsequent development of B-cell lymphomas [291]. Furthermore, cytokine dysregulation in macrophages, glial cells and astrocytes has been found to strictly correlate with neuronal dysfunction, brain injury and HIV dementia [149, 246, 266].

Recent discoveries in the field of HIV-1 coreceptors have provided new information about factors affecting disease progression (see section 1.2.6).

1.1.2 The structure and genome of HIV-1

HIV-1 is a member of the lentivirus genus of the *Retroviridae* family. The HIV-1 virion (Figure 1.1) is a spherical particle with a 90-130 nm diameter and a dense core [142].

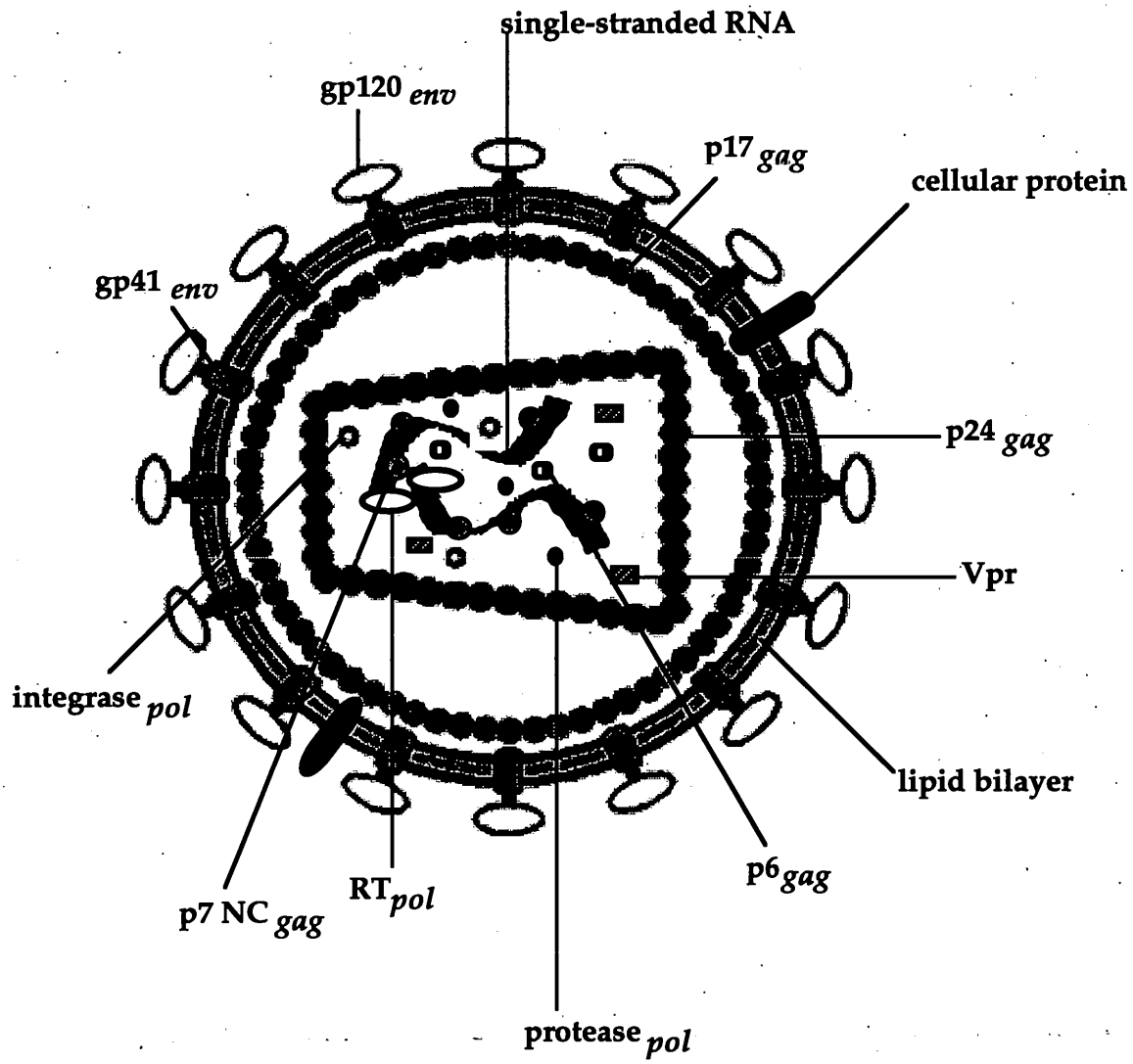

Figure 1.1 *Schematic representation of the HIV-1 virion*

The envelope of the virus consists of a lipid bilayer derived from the host cell membrane and two noncovalently associated subunits, gp120 and gp41, generated by cleavage of the gp160 precursor. The external gp120 glycoprotein derives from the N-terminal portion of gp160 and contains the CD4 binding site. gp41 is a transmembrane molecule derived from the C-terminal portion of gp160 and contains at its N-terminus a hydrophobic fusion peptide thought to be responsible for fusing the viral and target cell membranes [399]. The viral envelope glycoprotein (Env) that spikes from the surface of the virion or of

infected cells is a trimeric structure containing three gp120/gp41 complexes [385]. Most of the exposed surface of the assembled trimer is composed of elements of gp120, with the gp41 ectodomain largely buried inside the complex. Together with virus-encoded proteins, host-encoded transmembrane proteins, such as β2-microglobulin and MHC class I and II proteins, remain trapped in the lipid bilayer upon virion formation [12].

The viral genome consists of two identical copies of single-stranded RNA, each about 9.2 Kb long. Immediately after entry into cells, virion RNA is converted into double-stranded linear DNA that is subsequently integrated into the host cell genome as a provirus. The genome of HIV-1 (Figure 1.2), like that of all retroviruses, contains two identical long terminal repeats (LTR), that flank the viral genes, and contain discrete enhancer elements that bound and are controlled by several host transcription factors [83, 353].

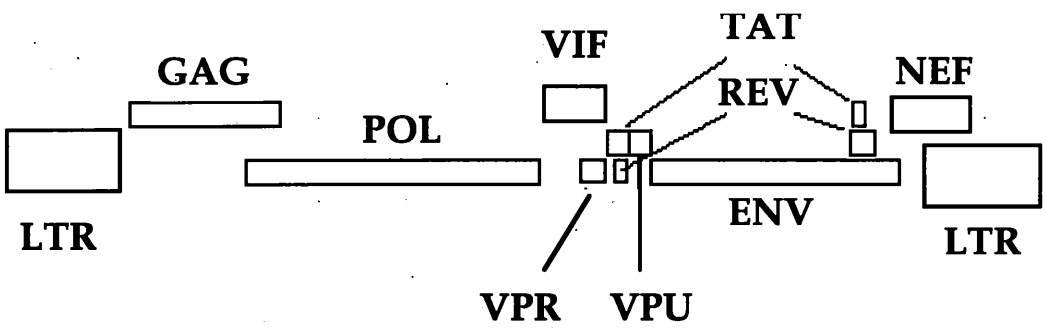

Figure 1.2 ***Genomic organization of HIV-1***

The 5′ LTR contains enhancer and promoter sequences essential for proviral transcription, whereas the 3′ LTR is required for transcript polyadenylation.

Like most retroviruses capable of replication, HIV-1 contains three main genes (*gag*, *pol* and *env*). The *env* gene encodes the precursor glycoprotein

gp160, which is cleaved into gp120 and gp41. The viral core is encoded by *gag* and includes p24 (capsid), p17 (matrix), p9 and p7 (nucleocapsid). The *pol* gene encodes the precursor for several virion enzymes, i.e., reverse transcriptase (RT), Rnase H, protease and integrase (IN) [118, 286]. Furthermore, the HIV-1 genome contains six additional genes (*tat*, *rev*, *vif*, *vpr*, *vpu/vpx* and *nef*) encoding viral proteins with regulatory or accessory functions [83, 365] (see below).

1.1.3 Replication cycle

The HIV replication cycle starts with the attachment and entry of the virus into target cells. HIV-1 entry is mediated by a high affinity interaction between the envelope glycoprotein gp120 and CD4 [85, 198]. In addition, gp120 uses members of the chemokine receptor family (described in detail in section 1.2) as coreceptors for entry. These interactions induce in gp120 a series of conformational changes [325] which trigger the exposure of the fusogenic portion of gp41. This in turn leads to pH-independent fusion between the virus and the host cell membranes [350, 385].

After fusion, the inner core of the virion is released into the cytoplasm and rapidly uncoated. The viral single-stranded RNA genome is converted into blunt-ended, double-stranded DNA by the enzyme RT. It is at this step that genetic variability appears, due to the errore-prone action of viral RT, which lacks proofreading ability. Viral DNA is thereafter transported into the nucleus as a preintegration complex which also contains the HIV-1 matrix and Vpr proteins, that regulate this process [49, 165]. At this point, integrase is required to integrate proviral DNA into the host genome. Integration of HIV-1, which occurs at random sites, seems to be essential for replication [319]. Non-

integrated circular forms of viral DNA are in fact presumably non-functional, dead-end products of the replication cycle [47].

Integrated proviral DNA may remain silent until the host cell is activated. Cellular activation by mitogenic or non-mitogenic stimuli and cytokines recruits to the 5′ LTR host transcription factors such as NFAT-1, USF, AP-1 and most importantly, NF-kB which - in combination with factors expressed under basal conditions (e.g., Sp1 and TFIID) - promote the full expression of HIV-1 genes [14, 139, 261]. LTR-driven gene expression can also be activated by proteins encoded by a variety of other viruses and retroviruses [23, 144].

The mRNA species encoded by HIV-1 can be divided into two classes based on their temporal expression. HIV-1 proviral transcription starts with the formation of multi-spliced RNA species (2 Kb) which encode the regulatory proteins Tat, Rev, and Nef [334] and represent the early class of mRNAs. Tat is a potent transactivator of HIV-1 transcription that acts via an RNA structure termed TAR (transactivation responsive region), located immediately 3′ to the LTR transcription start site [89, 124]. Tat increases the levels of viral transcripts and stabilizes the elongation of RNA transcripts that would be otherwise all prematurely terminated [82, 189]. Nef is required for efficient virus replication in primary lymphocytes and macrophages [90, 251, 348]. The late class of mRNAs consists of unspliced (9 Kb) and single spliced (4 Kb) transcripts encoding structural and accessory proteins [119, 194]. The expression of late mRNA species is dependent on the Rev protein. Rev binds to a complex RNA structure in the *env* gene, the RRE (Rev responsive element), and enables the nuclear export of unspliced and single spliced mRNA. Translation of the late transcripts by the cellular machinery gives rise to the HIV structural proteins.

The envelope precursor protein gp160 is glycosylated post-translationally and oligomerises in the endoplasmic reticulum (ER) before being incorporated into virions budding at the plasma membrane. During this transport, gp160 is cleaved into the gp120 and gp41 subunits. The Vpu regulatory protein is thought to enhance this process, and to inhibit the ER trapping of envelope proteins that would result from CD4 degradation [218, 391]. The myristoylated Gag and Gag-Pol precursor proteins aggregate at the inner surface of the cell membrane forming a spherical structure in which two copies of single-stranded RNA genome are encapsidated. The assembled core-RNA complex buds through the plasma membrane where it acquires the lipid layer, complete with viral envelope and host proteins [12, 142]. During this budding process, viral protease cleaves the Gag and Gag-Pol precursors into functional proteins, leading to the formation of a mature virion.

Among the HIV regulatory gene products, Vif is thought to play an essential role during virion maturation, since virions produced in the absence of Vif are less infectious [123, 320]. The Vpr protein is packaged into the virion nucleocapsid in molar amounts equivalent to those of Gag [69]. Vpr confers growth advantages to HIV-1 isolates expressing the protein, particularly in non-dividing cells such as primary macrophages. The myristilated Nef protein may also induce specific down-regulation of surface CD4 and MHC class I expression via endocytosis and lysosomal degradation. Through these mechanisms, Nef could hamper additional HIV infection and facilitate virion release from cells that express high levels of CD4 and would therefore trap virions on the cell surface. In addition, Nef can protect infected primary cells against killing by CTLs [1, 70].

1.1.4 HIV-1 variability

Several factors influence the considerable genetic diversity of HIV-1 that is one of its major characteristics. First, the error-prone nature of viral RT allows for nucleotide substitutions, deletions, insertions, duplications and recombinations. HIV-1 recombination occurs when two retroviruses containing different RNA strands infect the same cell, and the RT enzyme switches template during proviral DNA synthesis. Variation is amplified by the high viral turnover rate. It has been estimated that the virus half-life could be as short as 6 hours and that approximately 10^{10} new virus particles are produced and cleared every day [2, 10, 175, 382]. Finally, selective pressure allows for the rapid emergence of drug-resistant and immune escape mutants. Thus, heterogeneity is not confined to isolates from different individuals, and these factors continually drive the emergence of new virus variants within individual patients, giving rise to a population of closely related viral variants, commonly referred to as a quasispecies [162, 248]. Most commonly, during primary HIV-1 infection patients harbour a homogeneous population of viruses. With time, the viral population becomes increasingly heterogeneous. At later stages of the disease, the virus quasispecies may again become more homogeneous probably because individuals showing $CD4^+$ T cell decline and rapid progression to AIDS maintain a weaker immune response, resulting in a lower level of selection-driven changes in the virus pool [92, 153, 241, 411]. Compartmentalization of the quasispecies in different body organs has been documented in tissues such as brain, lung and testis, whereas lymphoid tissues usually harbour virus variants similar to those circulating in peripheral blood [375].

Genetic variation can arise throughout the HIV-1 genome. However, sequence variation is particularly high within the *env* gene, which is subdivided into five variable regions designated V1-V5 and interspersed with five more conserved regions termed C1-C5 [349, 399]. Mutations in the Pol and Gag proteins more often result in non-viable viruses, while env proteins tolerate extensive variation, which may actually represent a strategy to escape host immune surveillance.

Genetic analysis of viral sequences, predominantly from *env* and *gag*, has revealed that HIV-1 can be divided into three groups, major (M), outlier (O), and the recently found novel (N) [345]. Within the M group at least nine subtypes (A to H and J) have been identified [187, 260]. Inter-subtype variation has been shown to be approximately 20-30% for the *env* and 14% for the *gag* region, whereas the intra-subtype diversity is approximately 7-20% [136, 229]. Different subtypes exhibit different geographical distributions, but with the continuous spreading of HIV-1 it is becoming increasingly difficult to draw sharp borders. Subtype B viruses predominate in the North American continent, Europe and Australia. Both subtypes B and F are prevalent in South America, where subtype C has also been reported. Subtype G, as well as subtype H, has been isolated in Russia. Subtype E predominates in South Est Asia, while all group M subtypes, as well as group O variants, are found in Central Africa [36, 37, 136, 187, 388]. Cases of infection with two different strains of the same subtype, as well as two different subtypes (B and E) of HIV-1 have been reported. Moreover, chimeric viruses between two subtypes have been described.

1.1.5 HIV-1 phenotypes

HIV-1 genetic variation translates into a variation in biological properties, such as cell tropism, virulence and sensitivity to host immune responses. HIV-1 strains have been traditionally divided into two categories, based on their cellular tropism, replication kinetics, and ability to induce syncytia formation [56, 121, 356]. Virtually all HIV-1 strains replicate efficiently in $CD4^+$ peripheral blood lymphocytes (PBL). Many primary isolates are also able to grow efficiently in macrophages but not in immortalized T cell lines, and are thus termed macrophage-tropic (M-tropic). Based on the virus replication rate and the capacity to induce syncytia formation in T-cell lines, M-tropic viruses are also referred to as slow/low or non syncytia inducing (NSI). By contrast, T-tropic, rapid/high or syncytia inducing (SI) strains grow in T-cell lines, form syncytia in MT-2 cells and PBMC and tend to show higher replication rates and cytopathicity *in vitro*. The ability of SI isolates to productively infect macrophages has been controversial for some time (see section 4.2). Indeed, while T-cell line-adapted (TCLA) strains usually fail to replicate in monocyte-derived macrophages (MDM) [269], conflicting results have been reported when primary isolates (unselected by passage in cell lines *in vitro*) were used [75, 303, 326, 344, 373, 404].

The different HIV-1 phenotypes have been found to be strictly correlated with viral transmission and AIDS pathogenesis [74, 117, 156, 250]. Typically, upon transmission of HIV-1 between individuals, the NSI/M-tropic strains selectively take hold in the recipient and predominate during the asymptomatic phase of HIV-1 infection, which generally lasts for several years. SI/T-tropic strains are usually detected much later in many infected individuals, and their emergence is often associated with the rapid decline of

$CD4^+$ T cells heralding the demise of the immune system and the onset of AIDS [56, 121, 333, 357].

To map the viral determinants responsible for cell tropism, several laboratories analyzed chimeric molecular clones of T-tropic and M-tropic HIV-1 isolates [58, 185, 272, 337, 389]. The replication properties of these chimeras in different target cells led to the conclusion that the *env* gene (particularly a region of gp120 that includes the V3 loop) determines T- versus M-tropism. Subsequent studies confirmed the critical role of the V3 loop in the cell tropism of HIV-1, although interactions with other regions of gp120 (e.g. V1 and V2) also appear to be important [193, 202, 361]. However, it was only with the identification of HIV coreceptors that the mechanisms underlying HIV-1 cell tropism were fully unravelled.

1.2 CORECEPTORS FOR HIV-1 INFECTION

It had been known since the mid-1980s that CD4, a receptor expressed on the surface of T helper cells and macrophages, is required for high affinity binding of HIV Env to target cells, and therefore represents the major receptor for the docking of HIV on the membrane [85, 198]. However, later studies showed that expression of human CD4 on murine cells is not sufficient for HIV-1 infection [233]. Binding of gp120 to CD4-transfected murine cells could be demonstrated, yet virus entry did not occur. This phenomenon was observed also for HIV-2 and SIV and was shown not to be restricted to murine cells [63]. In fact, almost all non-human cells remain refractory to HIV-1 infection even when induced to express human CD4. Notably, nonpermissive $CD4^+$ cells can be made permissive for Env-mediated membrane fusion and infection by transient heterokaryon formation with HeLa cells, indicating that one or more components (or cofactors) in HeLa cells render non-human cells susceptible to HIV-1 infection [46, 99].

These results implied that HIV needs one or more factors other than CD4 for efficient entry into human cells. These cell surface molecules must be widely expressed in human cells of different lineages because, with rare exceptions [57], expression of CD4 on a variety of human cell lines renders them susceptible to HIV infection.

1.2.1 Chemokine receptors, the HIV coreceptors

Over the last 4 years it has become clear that the coreceptors required for HIV to infect $CD4^+$ cells are the chemokine receptors [5, 60, 93, 100, 120]. Central to this breakthrough were the findings of a study on the identity of soluble HIV

suppressive molecule(s) released by $CD8^+$ T cells. During the initial phases of HIV infection, the host immune system has the ability to restrain, albeit incompletely, virus spreading throughout the body [280]. $CD8^+$ T lymphocytes play an important role in controlling virus replication [329] and this immune control is mediated, at least in part, by soluble HIV suppressive factors produced by activated $CD8^+$ T cells [380]. The nature of such factors remained elusive for almost 10 years until Cocchi *et al.* reported the identification of three C-C chemokines - regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β - as major components of the HIV-suppressive activity released by both primary and *in vitro* immortalized $CD8^+$ T cells [67].

The experimental approach that lead to this discovery started with an analysis of the effect of supernatants from HTLV-I immortalized $CD8^+$ cloned T cell lines on the acute infection of a $CD4^+$ T cell line (PM1) with an M-tropic strain, HIV-1_{Ba-L}. Cell-free supernatants from clones with the highest HIV-suppressive activity were fractionated through a tangential flow filter and centrifugal concentrators. The concentrated fraction displaying HIV-suppressive activity in the PM1/ HIV-1_{Ba-L} test was further fractionated by weak anion-exchange high-performance liquid chromatography (HPLC). Fractions containing high levels of suppressive activity were pooled and further purified by reversed-phase HPLC. Potent HIV-suppressive activity, in the absence of significant cytotoxic effects, was recovered in separate fractions, each containing a single major protein peak. These proteins were each subjected to proteolytic digestion, followed by sequencing of distinct peptide fragments. The amino acid sequences revealed identity with three human C-C chemokines, RANTES, MIP-1α, and MIP-1β. Importantly, the HIV-suppressive activity expressed by

$CD8^+$ cells was completely blocked by a combination of neutralizing antibodies against these chemokines. When used as recombinant molecules, the three chemokines were active both alone and in combination, and they did not affect the viability nor the proliferative capacity of target cells.

RANTES, MIP-1α, and MIP-1β induced dose-dependent inhibition of the replication of different strains of primate lentiviruses (HIV-1, HIV-2, and SIV), whereas other RNA and DNA viruses, such as HTLV-I or human herpesviruses (HHV) 6 and 7, were not susceptible. Notably, chemokines were not equally effective against all viral strains: M-tropic/NSI HIV-1 isolates were potently inhibited, while T-tropic/SI isolates were insensitive.

The HIV suppressive activity of C-C chemokines strongly suggested that chemokine receptors might serve as coreceptors for HIV infection. This hypothesis was very soon confirmed by the identification of the CXC-chemokine receptor fusin (now designated CXCR4) as the coreceptor for the entry of T-tropic HIV-1 strains into $CD4^+$ cells. Feng *et al.* [120] devised a functional cDNA screening strategy to identify essential HIV coreceptors. Interestingly, this approach made no assumption about the nature of the cofactor(s) and was based on the ability of a cDNA library to render CD4-expressing murine cells permissive for fusion with cells expressing Env from a TCLA strain ($HIV\text{-}1_{IIIB}$). By repeated cycles of analysis, a single cDNA was isolated capable of conferring fusion ability to CD4-expressing murine cells. cDNA sequence analysis indicated that cell fusion mediated by the $HIV\text{-}1_{IIIB}$ Env was dependent on the expression of CXCR4/fusin, a member of the seven-transmembrane domain family of receptors highly homologous to CXC-chemokine receptors. The role of fusin as an HIV-1 coreceptor was further documented by demonstrating that coexpression of fusin and CD4 rendered

nonhuman cells permissive for Env-mediated cell fusion and infection, and anti-fusin antibodies potently inhibited fusion and infection of primary human $CD4^+$ T lymphocytes. As shown in figure 1.3, the natural ligands for CXCR4, unknown at the time, were later shown to be stromal cell-derived factor (SDF-1)α and SDF-1β, which are generated by alternative splicing of a single gene [33, 273].

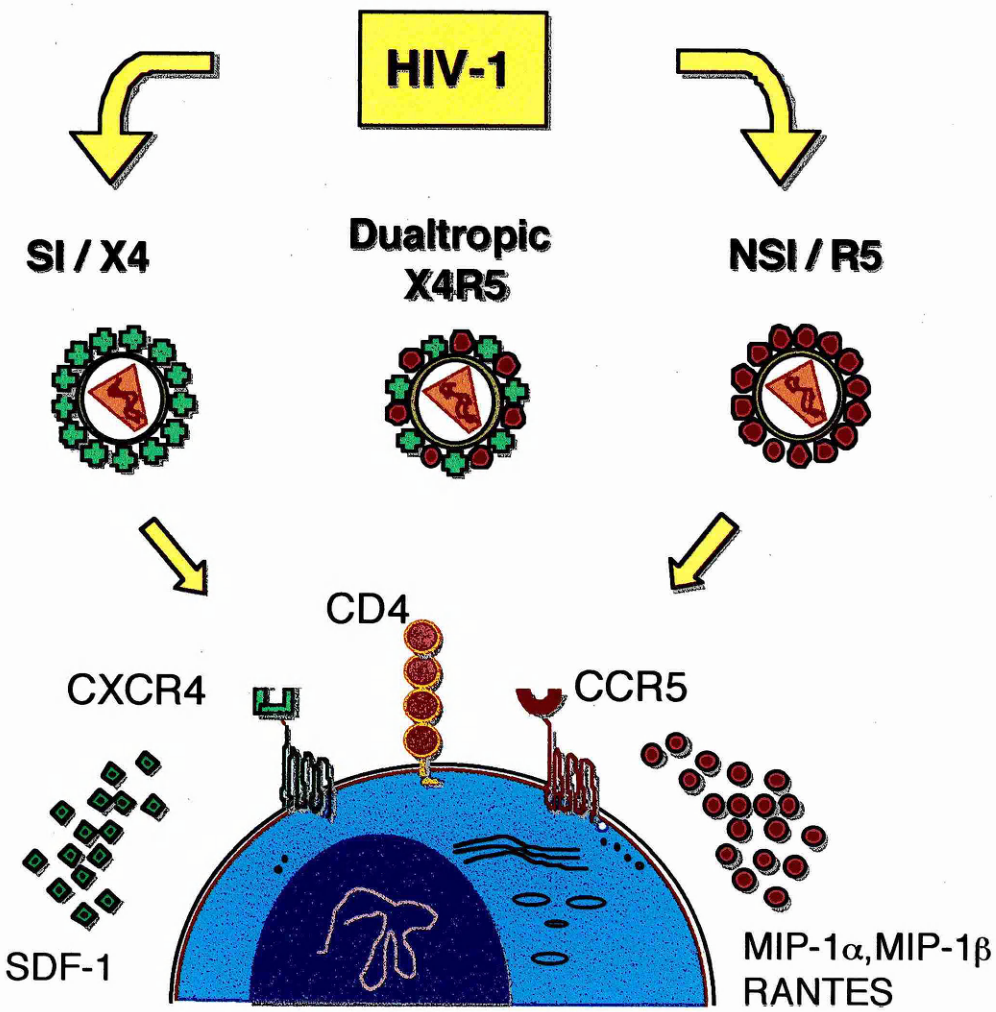

Figure 1.3 ***Chemokine receptors as coreceptors for HIV-1***

The work by Feng *et al.* showed that CXCR4 is the coreceptor for TCLA/SI HIV-1 isolates. However, CXCR4 was unable to mediate fusion of M-tropic HIV-1 isolates, whereas CC chemokines (RANTES, MIP-1α and MIP-1β) potently inhibited infection by M-tropic/NSI but not by TCLA/SI strains. This

discrepancy set the stage for the discovery of the second major coreceptor for HIV. Five different groups [5, 60, 93, 98, 100] simultaneously identified CCR5, the only C-C chemokine receptor known to selectively bind RANTES, MIP-1α and MIP-1β [323], as the main coreceptor for M-tropic HIV-1 isolates (figure 1.3). Similar to CXCR4, CCR5 was found to be essential for fusion between infected and uninfected cells; moreover, its expression induced susceptibility to HIV-1 infection, as assessed by virus production [5] or reporter gene expression [60, 93, 98]. CCR5 and CXCR4 are both expressed on known cell and tissue targets of HIV-1. In particular, CCR5 is expressed on monocytes, macrophages [72, 314], microglia, dendritic cells [157, 315], Langerhans cells [406], $CD4^+$ T cells [34], and the mucosa of rectum, colon, vagina and cervix [279, 409]. CCR5 levels on T cells are upregulated by interleukin 2 [228]. CXCR4 is expressed on virtually all hemopoietic cells.

In addition to CCR5 and CXCR4, several other chemokine receptors or structurally related molecules (listed in Table 1.1) [372] have been characterized as supporting HIV-1 Env-mediated fusion or viral entry *in vitro*. However, CCR5 and CXCR4 appear to be the coreceptors most widely utilized because in *in vitro* experiments all viruses use one or both receptors for entry. By contrast, CCR2b, CCR3, CCR8 and CCR9, the orphan receptors APJ, V28, STRL33, GPR1 and GPR15, and the human cytomegalovirus-encoded chemokine receptor homologue US28 function as coreceptors only for a limited number of viral strains [408, 410]. Moreover, it should be noted that *in vitro* studies on the role of different chemokine receptors in supporting HIV-1 entry are mostly performed using human cell lines that are stably transfected with both CD4 and individual potential coreceptors and express high levels of both molecules.

Therefore, it is not clear to what extent results obtained in such systems can be extrapolated to *in vivo* infection.

Alternative coreceptors for HIV and SIV				
Receptor	**Ligand**	**Expression pattern**	**HIV/SIV isolates**	**REF.**
CCR2b	MCP-1, MCP-2, MCP-3, MCP-4	Monocytes, T cells, B cells	HIV-1	4, 98, 127
CCR3	Eotaxin, MCP-4, MCP-3, RANTES	Eosinophils, Microglia, Th2 cells	HIV-1	5, 24, 60, 98 164, 313, 315 316
CCR8	I-309	Monocytes, Thymocytes	HIV-1, SIV	178
CCR9	TECK	Activated PBMC, Thymus	HIV-1	59
CX_3CR1 (V28)	Fractalkine/Neurotactin	Lymphocytes, Brain, NK cells, Monocytes	HIV-1, HIV-2	306, 316
APJ	?	Brain; Spleen	HIV-1,SIV	59, 106
GPR1	?	Macrophages	SIV	111
BOB/GPR15	?	T cells, Colon	SIV, HIV-2, HIV-1	94, 111
Bonzo/STRL33/ TYMSTR	CXCL16	T cells, Monocytes, Placenta	SIV, HIV-2, HIV-1	94, 224, 227
US28	MIP-1α, MIP-1β, RANTES, MCP-1	Monocytes, Lymphocytes in CMV-infected cells	HIV-1	290

Table 1.1 *Chemokine receptor family members that function as receptors for HIV and SIV entry*

Another relevant issue is the tissue expression pattern of potential coreceptors. Maintenance of high viral load is thought to require expression of coreceptors on activated $CD4^+$ T cells [285], but firm evidence to this effect is still lacking for many of the putative coreceptors. A significant hindrance to further studies is the unavailability of specific chemokine ligands and/or neutralizing monoclonal antibodies for many of these molecules. Thus, in spite of the large number of potential HIV coreceptors identified by *in vitro* studies, only CCR5

and CXCR4 have been established to play a major role in *in vivo* HIV infection.

Individuals homozygous for a 32 bp deletion in the CCR5 gene (*ccr5* Δ32) which results in the lack of CCR5 expression, are resistant to HIV-1 infection (see section 1.2.6) [182, 226, 324]. The existence of these "natural knockouts" for CCR5 suggests that CCR5 plays a fundamental role during HIV-1 transmission and replication *in vivo*. The physiological importance of CXCR4, on the other hand, is documented by the finding that HIV-1 isolates obtained from patients who experience a dramatic drop in circulating $CD4^+$ cells and rapid disease progression use this coreceptor efficiently *in vitro* [32, 76, 326, 344].

The expression and use of different coreceptors in cell types other than mature T lymphocytes and macrophages (e.g. cells in the brain or thymus) could contribute to some aspects of HIV-1 pathogenesis in both children and adults [26, 359]. Indeed, blockade of HIV-1 entry by the CCR3 ligand eotaxin indicated that CCR3 is an HIV coreceptor in microglia, although there is no consensus as to the relative importance of CCR3 and CCR5 for viral entry into these cells [26, 29, 148].

1.2.2 Chemokines, the natural ligands for HIV coreceptors

The discovery that chemokine receptors were the long sought coreceptors for HIV-1 infection resulted in a renewed interest in chemokines. These molecules represent a superfamily of 40 or more structurally related, low molecular weight (8- to 10-KDa) proteins that regulate migration and activation of mammalian leukocytes [17, 18]: hence their name, a contraction of chemotactic cytokines. Chemokines coordinate trafficking of different leukocyte subtypes to specific tissue destinations. Furthermore, they mediate

inflammatory responses by recruiting specific immune cells to the sites of inflammation, and inducing their activation [18].

Four classes of chemokines (CXC or α, CC or β, C or γ, and CX_3C or δ) can be defined based on the number and spacing of conserved cysteines. The CX_3C and C groups include only one known member and were identified only recently [25, 192], whereas more than a dozen members have been identified for the CC and CXC groups. In humans, the genes encoding CXC and CC chemokines are located in two distinct clusters on the long arms of chromosome 4 and 17, respectively. CXC chemokines are generally active on monocytes, neutrophils and peripheral blood T lymphocytes, whereas CC chemokines attract basophils and eosinophils, lymphocytes and monocytes [18]. Recently, it has also become clear that chemokines can be broadly divided into two categories (see Table 1.2): inflammatory, which are induced or strongly upregulated in peripheral tissues upon inflammation; and constitutive, which are involved in baseline leukocyte trafficking [16].

Chemokines induce leukocyte chemotaxis by binding to specific seven transmembrane domain G protein-coupled receptors [259]. There is redundancy in the system, i.e., most if not all chemokine receptors recognize more than one chemokine ligand, but they tend to be restricted by chemokine class. Ten human receptors specific for CC chemokines (CCR1-10) and five human receptors for CXC chemokines (CXCR1-5) have been identified by molecular cloning. Only one member of the family, the Duffy antigen receptor, is able to bind both CC and CXC chemokines [87, 268]. XCR1 binds the C chemokine lymphotactin, and CX_3CR1 binds the CX_3C chemokine fractalkine or neurotactin (reviewed in [257]).

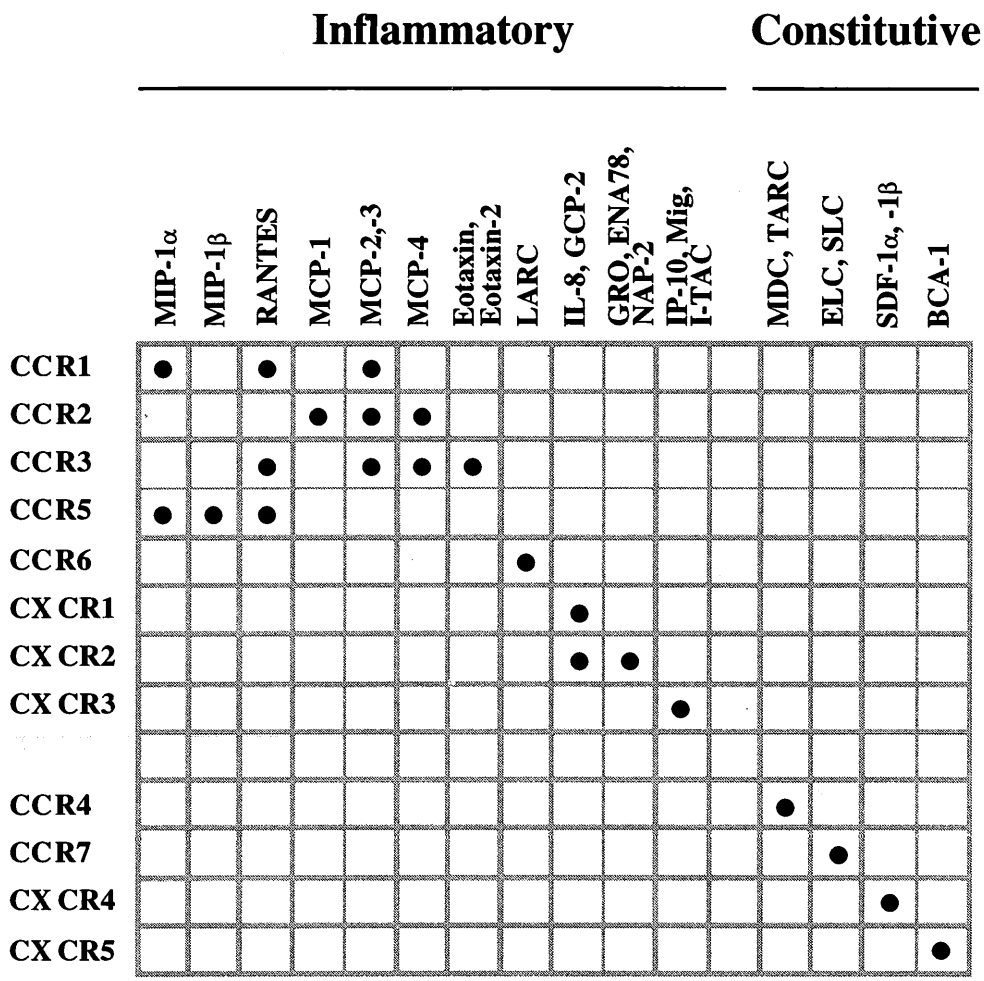

Table 1.2 *Human chemokine receptor-ligand specificity. Chemokine receptors are grouped according to their specificity for inflammatory or constitutive chemokines* (modified from Sallusto *et al.* Immunology Today 1998 19: 568-574).

The definition of the role of chemokines and chemokine receptors in HIV-1 infection has had two implications of great conceptual and practical relevance. On the one hand, chemokines may prevent viral infection by competing with viral envelope proteins for chemokine receptor occupancy. In addition and most importantly, the identification of chemokine receptors as HIV coreceptors has provided a molecular basis for the different tropism of

different HIV-1 strains, making it possible to associate viral phenotypes with specific coreceptor usage.

1.2.3 Chemokine receptors as determinants of viral tropism

For a long time, HIV-1 isolates were defined in terms of their cellular tropism (see also section 1.1.5). M-tropic/NSI viruses infect macrophages and primary T cells but not most immortalized T-cell lines. In contrast, T-tropic/SI viruses can infect primary T cells and T-cell lines, but their ability to infect macrophages has been controversial.

The molecular mechanisms underlying differences in both cellular tropism and phenotype among different strains of HIV-1 became apparent with the discovery that human chemokine receptors act as coreceptors for virus entry into $CD4^+$ cells (Figure 1.4). In particular CCR5, the RANTES, MIP-1α and MIP-1β receptor, has been shown to serve as the main coreceptor for M-tropic/NSI viruses [5, 60, 93, 98, 100] whereas CXCR4, the natural receptor for SDF-1 [33, 273], mediates the entry of SI HIV-1 strains, both primary and TCLA [120]. According to the most recent nomenclature based on receptor tropism, CCR5-tropic viruses are defined as R5, CXCR4-tropic viruses as X4, and dual-tropic viruses as R5X4 [27]. Studies of the expression of these receptors revealed that most T cell lines express high levels of CXCR4 and only rarely CCR5 [396]. However, while the lack of CCR5 expression on most T-cell lines provided a rationale for the inability of NSI strains to infect these cells, the ability of HIV-1 strains with an SI phenotype to infect macrophages was not clearly established. This issue is addressed in the *Results* (section 4.2) and in the *Discussion* (section 5.2).

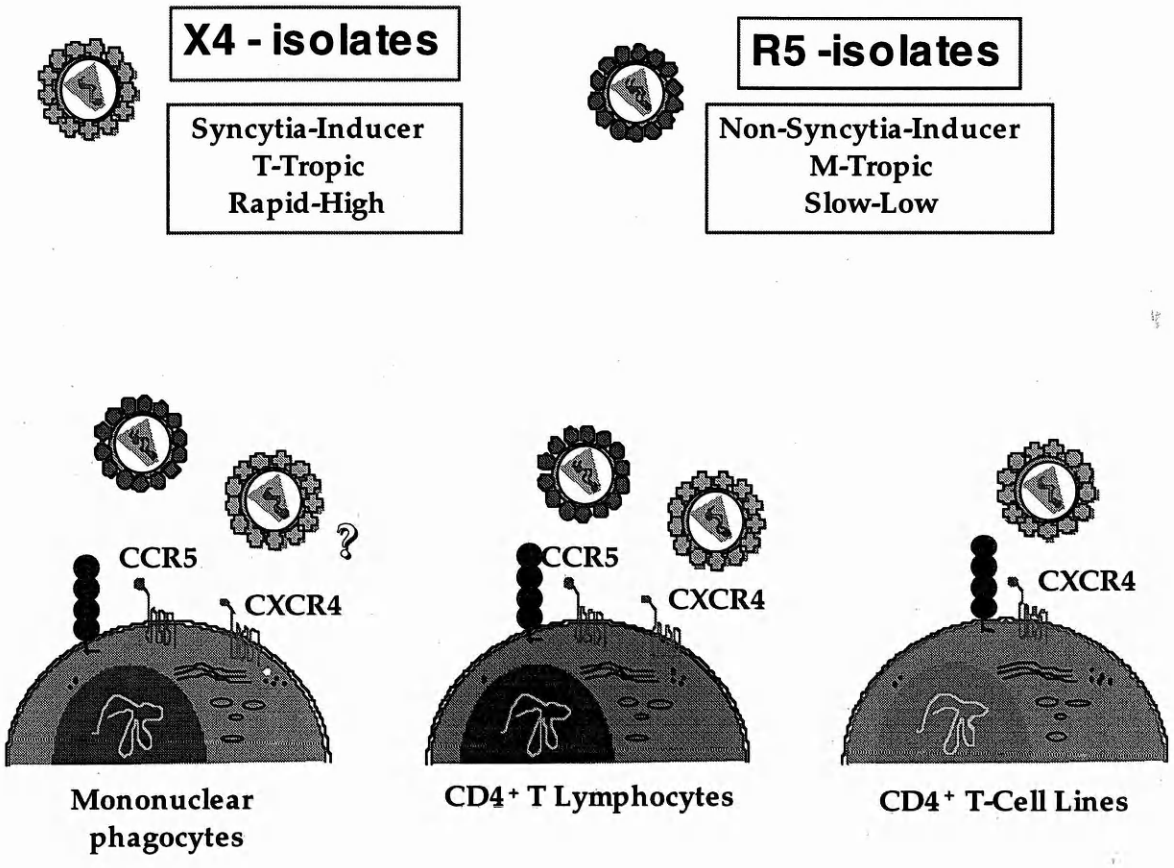

Figure 1.4 ***Biological variants of HIV-1 and their tropism***

1.2.4 Interactions between HIV, CD4 and the coreceptors

Biochemical studies using recombinant gp120, soluble CD4 and human or mouse cell lines transfected with chemokine receptors have addressed the interactions of gp120 molecules from R5 or X4 isolates with their specific coreceptors. Radiolabeled gp120 from R5 isolates binds CCR5-expressing cells only in the presence of added soluble CD4 [395]. HIV-1 and SIV envelope glycoproteins are able to compete with RANTES, MIP-1α and MIP-1β for CCR5 binding [172, 364, 395]. This competition was shown to be much more efficient in the presence of CD4, either co-expressed with CCR5 on the cell surface, or present in soluble form as a preformed complex with gp120. By contrast, soluble gp120 from X4 strains was found to form a precipitable complex with

CXCR4 [214], and to specifically bind CXCR4-expressing cells even in the absence of surface-bound or soluble CD4 [171]. However, the affinity of such interaction was clearly lower than that of the sCD4-HIV Env complex for CXCR4 [21]. Moreover, it has been shown that the association between CXCR4 and CD4 is enhanced in the presence of gp120, although it occurs to some extent also in its absence [369]. These findings suggest that the initial interaction of HIV-1 strains with CD4 induces a conformational change in gp120 which exposes, creates or stabilizes the subsequent binding of the envelope to the coreceptor. The binding of coreceptors to gp120 would trigger further conformational changes in the HIV-1 envelope, leading to exposure of regions of gp41 that mediate direct interactions with the lipid layer of the target cell membrane.

Recent papers have investigated determinants on gp120 responsible for tropism, Env-coreceptor interactions and fusion events. Results from these studies indicate that the V3 loop is critical for gp120 binding to coreceptor but, during the fusion process, V3 works in concert with other gp120 regions, including V1, V2 and C4 [209, 309, 398].

There are rare examples of situations in which CD4 is not even required for primate immunodeficiency lentiviral entry. This phenomenon was first described for HIV-2 strains that were selected for growth in $CD4^-$ cells and utilize either CXCR4, CCR5, CCR3 or the orphan receptor V28 [108, 305-307]. The same phenomenon was then observed for SIV [107, 237] and HIV-1 [103, 305]. Indeed, primary human fetal astrocytes, negative for CD4 and the major HIV-1 coreceptors CCR5, CXCR4, CCR3 and CCR2b, can be infected *in vitro* with HIV-1 isolates independently of coreceptor usage [318].

1.2.5 Structure-function studies on HIV coreceptors

All of the identified HIV/SIV coreceptors are members of the seven transmembrane domain G protein-coupled receptor family. As such, they contain an N-terminal extracellular domain, that is acidic and tyrosine-rich, and three extracellular loops (ECL 1-3) (Figure 1.5).

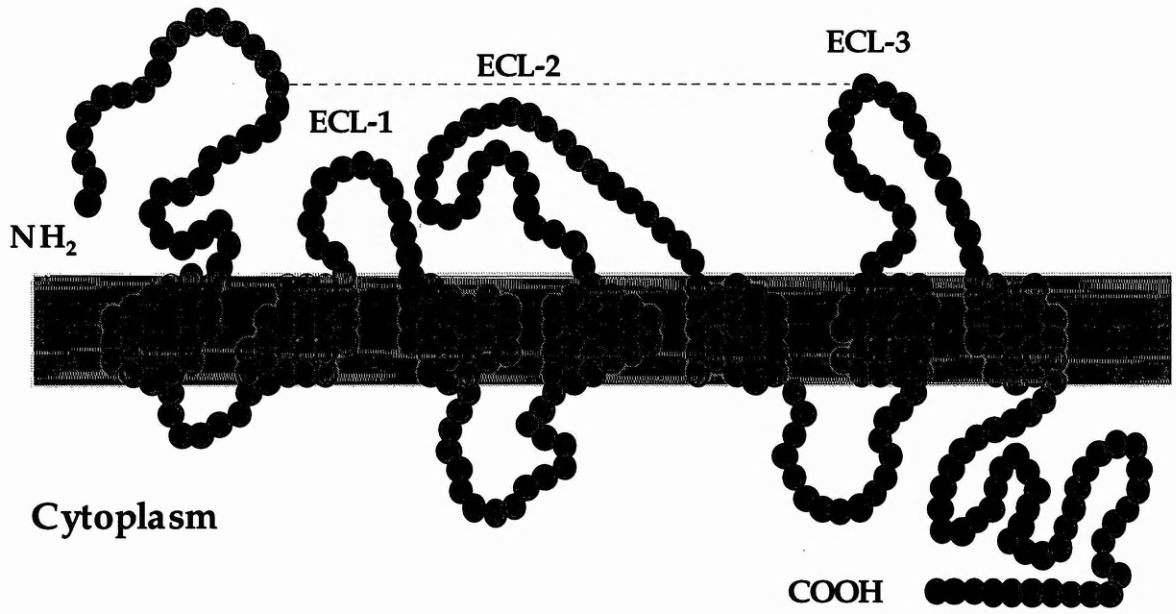

Figure 1.5 ***Schematic representation of a chemokine receptor***

Although they share a common structure, HIV coreceptors show marked variation at the amino acid level. Thus, structure-function studies have been performed to determine the region(s) needed by a seven-transmembrane-domain chemokine receptor to be a functional HIV coreceptor, and the amino acid residues that interact with the viral envelope. In addition, these studies have investigated whether the biological functions of G protein-coupled receptors (such as signaling and internalization) are required for HIV entry and infection.

Chimeric, mutant, and non-human homologous coreceptors have been constructed to identify the molecular determinants of CCR5 and CXCR4 coreceptor function. Structure-function studies of CCR5 suggest that multiple extracellular domains are involved in the activity of this coreceptor. Experiments performed on human/murine CCR5 chimeras showed that each of the extracellular domains of human CCR5 is independently capable of conferring some degree of coreceptor function to an otherwise inactive murine receptor [13, 30, 207, 287]. Notably, these chimeric coreceptors are often functional for a significantly more restricted range of HIV-1 strains, implying that different strains can interact with the coreceptors in different ways. Within this context, dual-tropic isolates appear to be particularly sensitive to perturbations in the human CCR5 extracellular regions [95, 302]. Functional redundance of the extracellular sites of CCR5 was also observed by studies on CCR5/CCR1, CCR5/CCR2b, CCR5/CXCR2 and CCR5/CXCR4 chimeras [97, 105, 230, 317]. Furthermore, mutational analysis of the extracellular domains consistently showed that residues important for coreceptor function are scattered throughout these regions, although several important residues are clustered within the N-terminus and ECL-2 [97, 101, 113, 302]. Of note, CCR5 is posttranslationally modified by sulfation of its N-terminal tyrosines. Sulfated tyrosines contribute to the binding of CCR5 to MIP-1α, MIP-1β and HIV-1 gp120/CD4 complexes and to the ability of HIV-1 to enter cells expressing CCR5 and CD4 [114].

Similar approaches to analyse structure-function relationships in CXCR4 led to the conclusion that multiple domains are involved in coreceptor activity. Unlike CCR5, the N-terminal domain of CXCR4 is considerably less important while ECL-1 and, particularly, ECL-2 appear to be critical for

coreceptor function [43, 230, 304]. These domains are also involved in SDF-1 binding, which could explain how this chemokine suppresses HIV infection.

Glycosylation could potentially modulate coreceptor utilization by creating and/or covering Env binding sites. CXCR4 has two potential N-linked glycosylation sites, one in the N-terminal domain and one in ECL-2, while CCR5 has one potential site in ECL-3. However mutation or elimination of these sites in both coreceptors has shown that they are not required for coreceptor function [43, 288, 317].

Activation of chemokine receptors by binding of their natural ligands leads to G protein coupling and subsequent intracellular signaling (reviewed in [298]). Therefore, during HIV infection binding of Env proteins to chemokine receptors could potentially initiate a signaling cascade affecting entry or post-entry events. To address this issue, conserved domains in the cytoplasmic region of CCR5 have been modified to prevent CCR5-mediated signaling [9, 112, 154]. Several studies indicate that signaling is fully dissociable from the role of CCR5 as an HIV coreceptor. Similar findings have been reported for CXCR4 [181, 230]. In addition, overexpression of arrestin (which enhances chemokine receptor internalization) or dominant negative arrestin mutants (which block chemokine-induced internalization) had no effect on the ability of CCR5 to act as an HIV coreceptor [9]. However, while signaling is not required for the entry of HIV-1 into target cells, it might be necessary for post-entry steps of the viral life cycle. Interestingly, monomeric or oligomeric gp120 molecules derived from several, but not all, M-tropic isolates could induce CCR5-mediated Ca^{2+} mobilization and chemotaxis in a proportion of activated $CD4^+$ T lymphocytes [387]. Moreover, gp120 molecules from various T-tropic or M-tropic isolates induce increased tyrosine phosphorylation of Pyk2 mediated

by an interaction with CXCR4 or CCR5 [88]. Finally, gp120 has been recently shown to specifically antagonize CXCR4 and CCR5 signaling in response to chemokines in a CD4-dependent fashion [232, 381]. These last results suggest that gp120 shed from virions and infected cells may bind to uninfected $CD4^+$ cells and affect the immune and inflammatory responses of infected individuals, thus contributing to the immunosuppressive effects of HIV-1.

1.2.6 Chemokine receptors in viral transmission and disease progression

The identification of chemokine receptors that function as coreceptors for HIV-1 entry into target cells lead to reconsider several open questions related to HIV-1 transmission and disease progression. As shown in Figure 1.6, examination of the phenotypes of HIV-1 strains sampled at different times during the course of infection revealed that recently infected individuals predominantly harbour R5 isolates, implicating the latter in viral transmission [81, 204, 374, 408]. Therefore, a major unanswered question is why X4 strains are rarely, if ever, transmitted, even though CXCR4 and other potential coreceptors are expressed on target cells, and X4 variants are present in the transmitting source. Indeed, X4 and R5/X4 isolates usually arise only late during infection and are prevalent during progression to symptomatic disease [32, 76, 326, 344]. However, it is not clear whether the emergence of CXCR4-dependent viruses is a consequence or rather the cause of the increasing immunosuppression, and what is the relationship between coreceptor usage and depletion of $CD4^+$ T cells. As shown in figure 1.6, a rapid decline in T-cell counts is temporally associated with a switch from R5 to X4 or R5/X4 variants. Experimental infection with R5 isolates causes less T-cell depletion than

infection with X4 or R5/X4 variants in T cell cultures, ex vivo infected lymphoid tissue [150, 283, 332], and SCID/hu mice chimeras [289], despite similar levels of viral replication. Interestingly, a recent paper demonstrated that R5 isolates are highly cytopathic, but only for $CCR5^+/CD4^+$ T cells [160]. Because these cells represent only a small fraction of $CD4^+$ T cells, their depletion does not substantially affect total $CD4^+$ T cell counts.

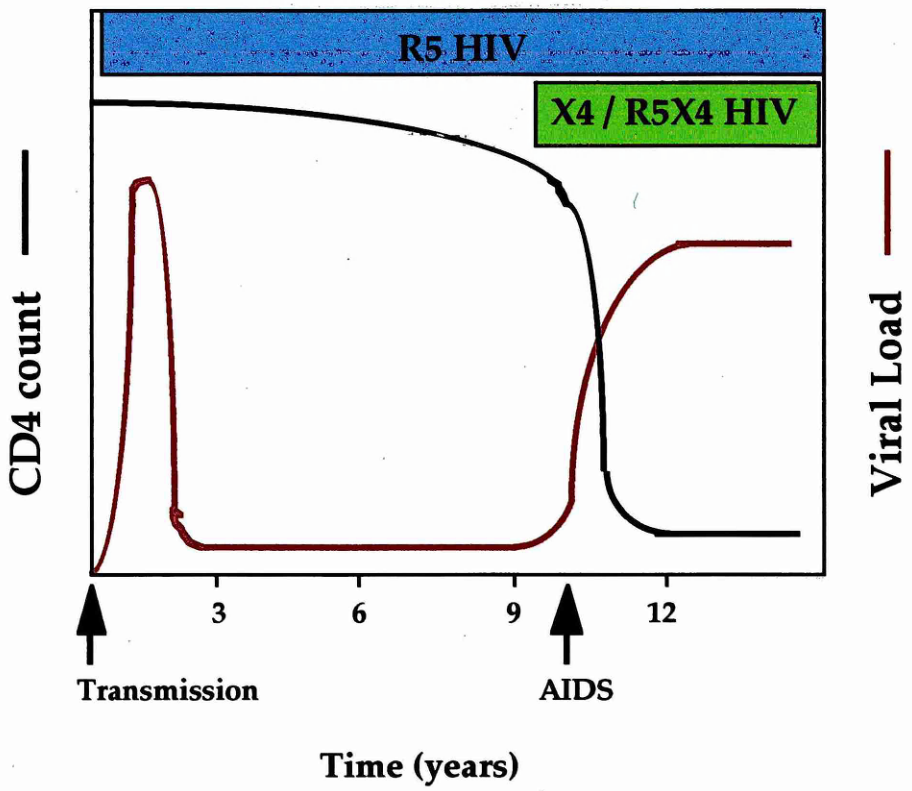

Figure 1.6 ***Temporal evolution of HIV-1 coreceptor usage during HIV-1 infection***

Because of important clinical and pathogenetic implications, it is essential to understand why some individuals who have been repeatedly exposed to HIV through high-risk sexual contacts with infected subjects remain uninfected, and why a small minority of infected persons are LTNP. The fundamental role of CCR5 during HIV-1 transmission *in vivo* is well

supported by the finding that some exposed uninfected (EU) individuals are homozygous for a 32 bp deletion in the coding region of the *CCR5* gene [226]. Notably, lymphocytes and macrophages isolated from these individuals were resistant to infection with R5 strains but readily infectable with X4 viruses [75, 281, 303]. The CCR5 sequence mutated in EU individuals encodes a region in the second ECL of the protein. The deletion results in a frame shift generating a severely truncated CCR5 protein which is not expressed on the cellular surface and rapidly degraded [91, 226, 324, 412]. About 20% of Caucasians are heterozygous, and 1% are homozygous for the Δ32 allele. In contrast, the latter was not observed in black African or Asian populations [225, 238]. Individuals homozygous for the Δ32 allele exhibit a normal immunological profile but are significantly protected against HIV-1 infection [91, 182, 324]. The Δ32/Δ32 genotype was found to be more frequent in several EU cohorts, including individuals exposed via mucosal or parenteral routes (homosexual men, intravenous drug users, and hemophiliacs). Indeed, only four individuals out of several thousand examined have been shown to be both Δ32 homozygous and HIV infected [20, 31, 271, 358]. In all cases, disease was progressive and the viral isolates appeared to be SI. In one of these individuals, X4 virus was exclusively and persistently detected [249].

Individuals heterozygous for the Δ32 allele are not highly resistant to infection [91, 104, 182, 324, 412]. However, the heterozygous genotype seems to confer a marginal benefit to individuals who become infected, as indicated by lower viral loads, slower rates of $CD4^+$ T-cell depletion, and increased times between seroconversion and progression to AIDS [247, 253]. All these observations suggest that during the initial phases of infection CCR5 is largely

responsible for HIV-1 transmission both between individuals and within the same individual, but the underlying mechanisms remain unclear. A crucial point is the expression pattern of different coreceptors on cells targeted by the initial infection (monocyte/macrophages, dendritic cells, Langerhans cells, vagina, cervix, rectum and colon). In this context, CXCR4 appear to be expressed at lower levels than CCR5 in colonic and cervical mucosa [279, 409].

Another issue under intense investigation is why viruses that use CXCR4 tend to arise only late in the course of infection, and what is their association with the dramatic drop in $CD4^+$ T cell counts. It has been proposed that the evolutionary pressures that may select for a shift in chemokine receptor utilisation could involve expression of inhibitory chemokines [366], depletion of specific target cells and/or escape from immune responses. In fact, the potential for using multiple members of the chemokine coreceptor family for cell entry may provide a convenient strategy for the virus to escape a potent neutralising host antibody response directed against regions (such as the V3 loop) involved in specific interactions with receptors. Moreover, it has been hypothesized that CXCR4-dependent viruses become prevalent late in disease as a consequence of the selective depletion of $CD4^+$ memory T cells, the subset that preferentially expresses CCR5. These are replaced at high rate by naive T cells which express preferentially CXCR4 [34], thus providing a selective advantage for X4 isolates. However, it is important to mention that the occurrence of SI/X4 isolates in only about 50% of patients with advanced disease indicates that the ability of the virus to use CXCR4 is not an absolute prerequisite for the onset and progression of AIDS.

LTNP are patients who, although infected, show no disease symptoms for 10-15 years or longer. Some of them appear to have been infected with

highly attenuated viral strains, such as those bearing deletions in *nef*. However, the majority of LTNP appear to be infected with isolates that bear no obvious genetic defects. In these cases, it has been suggested that a peculiar genetic background of the individuals may underlie their relative resistance to disease progression. Recently, some polymorphisms have been identified in coreceptor and/or chemokine genes, and correlated with delayed HIV-1 disease progression:

- A heterozygous mutation in *CCR2b* [346] has been shown to correlate with delay in the onset of disease. The protective effect of a single amino acid change (V64I) in the first transmembrane domain of *CCR2b* is unexpected because most HIV-1 strains are unable to use CCR2b as a coreceptor. However, the *CCR2b* polymorphism appears to be invariably associated with a polymorphism in the closely linked *CCR5* promoter, the functional impact of which remains to be demonstrated. CCR2-64I has no effect on initial HIV transmission. However seroconvertors bearing the *CCR2-64I* allele tend to progress to AIDS 2-3 years later than CCR2 +/+ HIV seroconvertors. Moreover CCR2-64I is enriched among LTNP and reduced in rapid progressors [206, 308, 346].

- A single nucleotide polymorphism in the CCR5 promoter (CCR5 59029 G/A) is associated with a 3-4 year delay in mean progression time to AIDS [239].

- Consistent with the critical importance of chemokines in HIV-1 pathogenesis, homozygosity for a polymorphism in the 3' untranslated region of SDF-1 (SDF1-3'A), the CXCR4 ligand, confers long-term protection against disease progression [393]. However, a clear role of SDF1-3'A in HIV-1 disease has not been documented because a similar study on a different cohort

reported that this polymorphism was actually associated with accelerated disease progression [255].

1.2.7 The therapeutic potential of chemokines and chemokine receptors

The identification of chemokine receptors that mediate HIV entry has obvious therapeutic implications. Indeed, chemokines are natural inhibitors of HIV entry. In particular, NSI/R5 viruses are inhibited by CCR5 ligands, such as RANTES, MIP-1α and MIP-1β [67], and SDF-1 inhibits entry of SI/X4 or dual-tropic HIV-1 viruses that use CXCR4 [33, 273]. Likewise, infection by NSI viruses that use CCR3 as a coreceptor is inhibited by eotaxin, the main CCR3 ligand [164], and I-309 inhibits CCR8-dependent infection by different HIV-1 strains [178]. In addition, C-C chemokine homologues encoded by Kaposi's sarcoma-associated herpesvirus have been reported to block HIV infection [40, 199].

Although the use of chemokines as anti-viral drugs may be complicated by their normal biological activity, these results have opened the way for a number of novel antiviral approaches. Individuals homozygous for the Δ32 allele in the CCR5 gene exhibit a normal immunological profile, [226, 324], suggesting that antagonists targeted specifically to this coreceptor would not have undesirable effects. The search for specific chemokine receptor antagonists is ongoing and involves the design of protein antagonists and peptide inhibitors. A number of studies have demonstrated that N-terminal modification and truncation of chemokines gives rise to specific receptor antagonists. This approach has been used to create two RANTES antagonists that display a potent inhibitory effect during infection with R5 strains in both

macrophages and lymphocytes [11, 231, 341]. Both proteins are N-terminally modified, have a high affinity for CCR5, lack chemoattractant activity and block RANTES-induced chemotaxis. By contrast, the design of CXCR4-targeted antagonists has to take into account the evidence that SDF-1 knock-out mice have lethal defects in B cell lymphopoiesis [262]. Nevertheless, it may be possible to develop small inhibitors that block viral interactions with CXCR4 without compromising SDF-1 signal transduction. Two reports have described peptide inhibitors of CXCR4 that block HIV-1 infection [96, 256]. The first, known as T22, is an 18-residue oligopeptide, the second, known as ALX40-4C, is a highly cationic oligopeptide containing nine Arg residues. Both molecules specifically inhibited CXCR4-mediated membrane fusion and viral entry by X4 isolates. In addition, ALX40-4C blocks SDF-1-dependent activation of calcium mobilization and binding of the CXCR4-specific monoclonal antibody 12G5. However, the therapeutic potential of these peptides is somewhat limited by their relatively poor metabolic stability. Recently, AMD3100 a heterocyclic bicyclam compound previously reported to block HIV-1 replication, was shown to inhibit binding of SDF-1 and 12G5 antibody to CXCR4, and to neutralize CXCR4-dependent viral infection [331]. However, all these compounds also block signaling through CXCR4 and it is not known whether they will have adverse effects on the host.

Chemokines exert two types of anti-HIV activities, competition for HIV-1 binding to chemokine receptors and downregulation of surface coreceptor expression. These processes are functionally linked since occupancy of the receptor triggers its internalization [6, 7, 231]. Thus, a reduction in the level of surface expression of viral coreceptors could represent an alternative strategy to inhibit HIV infection. To obtain cell-surface receptor depletion one group has

devised a mechanism to trap CXCR4 and CCR5 in the ER, thereby preventing their transport to the cell membrane [54, 400]. The tetrapeptide sequence KDEL, which is an ER retrieval sequence, was engineered onto the C-terminal end of MIP-1α, RANTES and SDF-1. These intracellularly retained chemokines - called "intrakines" - can be transduced into lymphocytes where they prevent surface expression of newly synthesized CCR5 and CXCR4, probably by forming intracellular complexes. Gene therapy approaches have also been proposed to target CCR5 mRNA using ribozymes or antisense oligonucleotides [151]. Finally, fusion-competent HIV vaccine immunogens were generated that capture the transient envelope-CD4-coreceptor structures that arise during HIV binding and fusion. In a transgenic mouse immunization model, these formaldehyde-fixed whole cell vaccines elicited antibodies capable of neutralizing infectivity of 23 out of 24 primary HIV isolates from diverse geographic locations and subtypes A to E [211].

An important consideration in the development of therapeutic strategies based on coreceptor usage is the possibility that inhibitors of individual coreceptors could select for strains that use an alternative coreceptor or different regions of the same coreceptor. In this respect, it is even possible that CCR5-targeted therapy may accelerate the course of the disease by selecting for viral strains that use CXCR4 [196] or perhaps CCR2b, or CCR3, the emergence of which is associated with disease progression [76, 326]. These observations warrant the conclusion that a combination of agents aimed at abolishing the coreceptor function of both CCR5 and CXCR4 may be needed to successfully affect the propagation of HIV-1 *in vivo*.

1.3 HIV-1 INFECTION IN HUMAN MACROPHAGES

HIV-1 infection requires access to host cells that are susceptible to and capable of replicating the virus. As emphasized above, cellular susceptibility is dependent upon membrane expression of CD4 and the chemokine receptors, mostly CCR5 and CXCR4. Thus the major cell lineages that are susceptible to infection *in vivo* are $CD4^+$ T lymphocytes and monocytes/macrophages. Macrophages play an important role in HIV transmission and propagation of viral infection. Macrophages can be found in the lamina propria adjacent to mucosal epithelium, and in cases of genital ulceration or lesion in the mucosal epithelium, they may come in direct contact with HIV [270, 296]. Thus, macrophages represent the predominant cell type infected with HIV in tissues [147, 201, 321]. HIV-infected macrophages are found in brain, lung, lymph nodes, and skin of seropositive patients and are likely to directly contribute to HIV-induced immunosuppression, central nervous system dysfunction and development of pulmonary complications. Indeed, HIV-2/SIV_{SM} variants that infect macrophages inefficiently *in vitro* were considerably impaired in their replication capacity and pathogenicity *in vivo* [174].

Circulating monocytes show low levels of infection as assessed by DNA polymerase chain reaction (PCR), indicating that infection may occur after the cells have left the circulation and differentiated into macrophages [330]. In *in vitro* infections, macrophages are relatively resistant to the cytopathic effects of HIV-1. This observation led to the suggestion that infected macrophages may represent a major reservoir for the virus *in vivo*, contributing to the spread of virus to different tissues within infected patients and between individuals. Macrophages play also an important role in the presentation of antigens to

$CD4^+$ T cells, and it is possible that infected macrophages may transmit the infection to $CD4^+$ T cells during this process [234].

In addition, macrophages modulate immune responses and tissue functions through the release of a large array of secretory molecules [267]. Changes in the secretion of cytokines and mediators occur during HIV infection and underlie the symptomatology of AIDS [116, 117, 347].

1.3.1 The viral cycle in macrophages

The HIV-1 life cycle in macrophages differs in many respects from that in T cells. Indeed, macrophages are terminally differentiated cells and, under normal conditions, they do not proliferate. Although retroviruses tipically infect only dividing cells [222], the action of several primate lentiviral proteins allows for efficient infection of nondividing macrophages. Thus, unlike T cells, a productive HIV-1 infection in these cells occurs independently of cellular DNA synthesis [141, 383].

Infection of susceptible macrophages can be mediated by CD4 and chemokine receptors, as well as by an alternative route. The observation that *in vitro* infection of macrophages with HIV was markedly enhanced (5- to 10-fold increase in RT activity) by sera from certain HIV-infected patients [310] suggested that virus-antibody complexes might bind to Fc receptors on the surface of macrophages and subsequently enter the cells via endocytosis. This mechanism, known as antibody-dependent enhancement (ADE), occurs in several human and animal viral diseases, including those caused by lentiviruses [52, 265]. It has been shown that ADE of HIV infection in macrophages is mediated by FcγRIII (CD16) in a CD4-independent fashion [176, 363].

Analysis of HIV-infected macrophages by transmission electron microscopy suggests another fundamental difference between virus-macrophage and virus-T cell interactions. In HIV-infected lymphocytes, the assembly and budding of viral particles takes place on the cytoplasmic membrane. By contrast, few or no virions are found at the plasma membrane in macrophages. Yet these infected cells contain large numbers of viral particles which are localized almost exclusively in intracellular vacuoles that probably belong to the Golgi apparatus [35, 276].

Distinct effects of HIV-1 accessory genes were also observed in primary macrophages and lymphocytes. Mutational analysis of primary HIV-1 isolates showed that mutant viruses had markedly different patterns of replication in macrophages, whereas differences were modest in lymphocytes. Indeed, loss of Vpr or Vpu reduced viral antigen production in macrophages as much as 1000-fold, while replication in T cells was only marginally affected [19, 73]. Current consensus suggests that HIV-1 Vpr is a positive regulator of viral replication in macrophages. Most retroviruses fail to infect nondividing cells, as mitosis and conceivably the disintegration of nuclear membrane that occurs during cell division are prerequisites to their gaining access to the cell nucleus. However, in HIV-1, Vpr and at least two other virion proteins (matrix p17 and IN) contribute to the nuclear import of proviral DNA in growth-arrested cells, such as macrophages [48, 131-133, 165]. The efficient translocation of proviral DNA into the cell nucleus is an active process driven by the interaction between an array of nuclear localization signals (NLS) present on matrix protein, IN, and Vpr and the cellular NLS receptor Karyopherin alpha [378]. In addition, Vpr has been reported to increase viral transcription in macrophages [354]; thus, the role of Vpr appears to be essential rather than accessory in this

cell population, as it combines the ability to confer optimal infectivity (early function) with the ability to augment viral production after infection (late function).

After HIV enters the host cell and proviral DNA becomes integrated into the host genome, transcription of viral RNA is dependent on cellular proteins. Some cellular transcription factors required for HIV-1 transcription, such as Sp1 and NF-κB, are ubiquitously expressed while others, such as GATA-3, ETS-1, LEF-1, and NF-ATc are lymphoid- or T cell-specific [159, 195, 336, 402]. Recent studies demonstrate that there are three C/EBP binding sites within the HIV-1 LTR and that C/EBP proteins are necessary for HIV-1 replication in macrophages but not in T cells [166-168]. C/EBPβ is usually induced upon activation of macrophages and activates transcription of many endogenous genes whose products are important for monocyte/macrophage function including the cytokines IL-1β, IL-6, IL-8, granulocyte-colony stimulating factor, and the chemokine MIP-1α. Thus HIV-1 takes advantage of the mechanism used by monocyte/macrophages to coordinate the transcription of highly expressed endogenous genes. In addition, many of the cytokines produced by macrophages can further upregulate viral replication by inducing NF-κB and C/EBPβ, and these 2 factors can interact synergistically. Thus, C/EBPβ plays a central role in an autostimulatory pathway involving macrophages, cytokines and HIV-1 infection.

1.3.2 Role of macrophages in the pathogenesis of HIV-1 infection

Evidence from different lines of work highlights the deleterious role played by macrophages in HIV-1 disease. Unlike HIV-infected T cells, HIV-infected macrophages appear to be resistant to the cytopathic effects of the virus and

thus serve as a reservoir for persistent infection and virus dissemination. Furthermore, macrophages may serve as sites for virus replication late in AIDS when T cell numbers are low, or following withdrawal of treatment with viral inhibitors [53, 115, 141, 175, 284, 285]. In fact, the presence in the body of persistently infected macrophages represents a key challenge for therapeutic efforts to eradicate HIV infection by eliminating all cells harboring the viral genome and/or sustaining virus replication for a long period of time. Nerve growth factor (NGF) has been recently reported to be an autocrine survival factor that rescues human macrophages from the cytopathic effects of HIV infection [138]. Thus macrophages in spleen, lymph nodes, bone marrow, liver, and other tissues may take advantage of their autocrine NGF, survive and continuously produce viral particles. This may be particularly relevant in the central nervous system, where macrophages represent the majority of HIV-infected cells, and most resident cells are able to produce NGF.

HIV-1 infection of human macrophages is associated with increased apoptosis of $CD8^+$ T lymphocytes. These cells are known to play an important role in the control of infection through their cytotoxic activity and the release of soluble HIV suppressive factors. In AIDS patients, the absolute number of $CD8^+$ T cells is decreased in peripheral blood and their turnover rate is increased. Apoptosis in this cell population seems to be triggered by the interaction between TNF-α on the membrane of macrophages and TNF-receptor 2 expressed on $CD8^+$ T cells. This phenomenon is indirectly mediated by the engagement of CXCR4 that upregulates cell surface expression of both molecules specifically in those cell subsets [170].

Another contribution of macrophage lineage cells to virus replication and pathogenicity resides in the ability of the accessory gene product Nef to

induce production of C-C chemokines, particularly MIP-1α and MIP-1β, by HIV-1-infected macrophages. Secretion of these chemokines promotes chemotaxis of resting T cells (which are normally refractory to productive infection) to sites of virus production, thus facilitating virus dissemination from infected macrophages to T lymphocytes [355].

Finally, one of the most important involvements of macrophages in HIV pathogenesis is associated with the emergence of opportunistic infections (OIs). The ability of HIV to infect and disable T cells renders the host susceptible to a broad range of opportunistic viral, bacterial, fungal, and protozoal pathogens that mark the progression of HIV disease. Infection with common opportunistic pathogens, such as HHV-1, *Mycobacterium avium* complex, and *Mycobacterium tuberculosis*, is accompanied by increases in HIV-1 viremia [146, 152, 169, 362]. Conversely, therapeutic intervention for treatment and prevention of a wide variety of OIs reduces viremia and consequently morbidity and mortality in AIDS. Analysis of lymphoid tissues co-infected with HIV-1 and OIs revealed that cells of the monocyte/macrophage lineage become the major source of the dramatic increase observed in HIV-1 levels. Remarkably, pathogens have the capacity to establish foci of HIV production by recruiting HIV-1-infected and infectable macrophages and stimulating their viral expression [275].

1.3.3 Macrophages and bacterial infections

Besides their important role in both the natural history and the pathogenesis of HIV infection, monocyte/macrophages are critically involved in the response to bacterial infections. Lipopolysaccharide (LPS)/endotoxin, the major constituent of the Gram-negative bacterial cell wall, activates multiple

macrophage effector functions that serve to coordinate host protective immune and inflammatory responses. However, increased exposure to LPS can lead to septic shock, a serious syndrome characterized by tissue injury, circulatory collapse, multiorgan failure, and death [39, 352]. LPS stimulates host cells, particularly monocytes and macrophages, to produce and release endogenous mediators including the proinflammatory cytokines IL-1, IL-6, and TNF [371]. The effects of LPS are mediated by CD14, a glycosylphosphatidylinositol (GPI)-anchored membrane protein specifically expressed on cells of monocyte/macrophage lineage [215, 394], and Toll-like receptor (TLR) 2 [197, 401] and TLR4 [295, 301] that act as signal transducers. Interestingly, CD14 serves as a recognition molecule for a wide variety of bacterial wall molecules, such as mycobacterial lipoarabinomannan (LAM) and components of Gram-positive bacteria [299]. Thus, CD14 is a pattern recognition receptor with multiple microbial ligand-binding specificities and for this reason belongs to the set of nonclonal immune receptors highly conserved throughout evolution, and responsible for nonadaptive (innate) immunity [242].

2 Aims of the thesis

Macrophages play a key role in HIV-1 infection. On the one hand, these cells are essential for the pathogenesis of HIV-1 disease because they are among the first targets infected by the virus *in vivo*, and represent a major reservoir for HIV-1 at all stages of infection. Furthermore, unlike T cells, macrophages are resistant to the cytopathic effects of HIV-1. Infected macrophages may therefore persist in tissues for long periods of time, providing a vector for the spreading of infection to different tissues. In addition macrophages are thought to play a regulatory role in controlling disease progression through the release of inflammatory cytokines.

On the other hand, macrophages are critically involved in the immune response to bacterial infections. LPS or LAM released by bacteria or mycobacteria evoke strong inflammatory responses by inducing macrophages to secrete cytokines and chemokines. This process is mediated by the engagement of CD14, a pattern recognition receptor for foreign lipoglycans expressed at high levels on monocytes and macrophages.

Patients with HIV-1 infection are immunosuppressed, often severely, and thus become highly susceptible to bacterial superinfections. Indeed, LPS can reach significant levels in the blood and liver of these patients. Therefore it was important to determine whether and how bacterial products modulate HIV-1 replication. Interestingly, LPS/CD14 interactions upregulated HIV-1 expression in monocytoid tumor cell lines [15, 297], but protected primary macrophages from productive infection by HIV-1 *in vitro* [205].

The work presented herein was aimed at a characterization of HIV-1 infection in primary macrophages, and particularly at a dissection of the mechanisms responsible for the LPS-induced inhibition of HIV-1 replication.

Initial experiments investigated the effects of LPS on the replication of R5 HIV-1 isolates in cultures of monocyte-derived macrophages (MDM) isolated from normal donors and infected *in vitro*.

While this work was in progress, the identification of chemokine receptors as the coreceptors for HIV-1 entry into CD4+ cells provided a molecular basis of the different cellular tropism of different HIV-1 strains. However, the ability of X4 viruses to productively infect macrophages remained controversial. Because of the implications that susceptibility to infection with these viruses has for the role of macrophages in disease transmission and progression, this issue was addressed by investigating whether CXCR4 is a functional HIV-1 coreceptor in MDM.

The final part of this work was predicated on the finding that primary X4 HIV-1 isolates indeed infect human macrophages, and was aimed at assessing whether the protection conferred to macrophages by LPS stimulation extends to infection by CXCR4-dependent HIV-1 isolates.

3 Materials and Methods

3.1 Reagents

Monoclonal antibodies (mAbs) specific for human CXCR4 (12G5) and CCR5 (2D7) were kindly provided by J. Hoxie and Leukosite Inc., respectively, through the AIDS Reagent Project, National Institute for Biological Standards and Control. Anti-CD4 mAb Leu 3A, anti-CD14 mAb P9, FITC-conjugated goat anti-mouse IgG and isotype controls were purchased from Becton Dickinson (Mountain View, CA). rTNF-α, recombinant C-C chemokines (RANTES, MIP-1α, and MIP-1β) and neutralizing goat polyclonal antibodies against IL-1 receptor antagonist (IL-1Ra: neutralizing dose, ND_{50} = 5-10 μg/ml), MIP-1α (ND_{50} = 10 μg/ml), MIP-1β (ND_{50} = 40 μg/ml), and RANTES (ND_{50} = 100-200 μg/ml) were obtained from R&D Systems (Minneapolis, MN). Neutralizing sheep polyclonal antibodies against IFN-α and IFN-β were obtained from Biosource International (Camarillo, CA). rSDF-1β was obtained from Upstate Biotechnology (Lake Placid, NY). A neutralizing rat anti-human IL-10 mAb (J53-19F1, IgG2a) was a kind gift of Dr. J. Abrams (DNAX Research Institute, Palo Alto, CA). The mAbs used in the ELISA assay for soluble TNF receptor 1, and in the immunofluorescence analysis of membrane TNF-α expression were kindly provided by Dr. A. Corti (San Raffaele Scientific Institute, Milan, Italy). Concentrations of TNF-α, IL-6, MIP-1α, MIP-1β and RANTES in culture supernatants were determined by ELISA (Quantikine, R&D Systems). Recombinant IFN-α (specific activity: 3.75 U/ng) was a kind gift from Roche Milano Ricerche. IFN-α concentrations in macrophage supernatants were assessed by ELISA (Biosource). LPS from S. *minnesota* and purified goat IgG

were purchased from Sigma Chemical Co. (St. Louis, MO). Polymyxin B, sulfate was purchased from Calbiochem (La Jolla, CA).

The endotoxin content of all cell culture reagents was assessed by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD), and was always <0.125 EU/ml.

3.2 Characterization of viral co-receptor usage

Co-receptor usage was characterized using the human glioma cell line U87.CD4 coexpressing CCR1, CCR2B, CCR3, CCR5 or CXCR4 [326] and osteosarcoma GHOST34.CD4 cells transfected with the Bob/gpr15 or Bonzo/STRL33/TYMSTR genes (kindly provided by D.R. Littman, Skirball Institute, New York, NY). Cells were seeded in 24-well plates at $5x10^4$ cells/well in DMEM-10% FCS and infected 24 hours later with primary isolates and TCLA strains (5 ng of virus) overnight. All cell cultures were observed daily for cytopathic effects, and p24 Ag secretion was assessed every 3 days during 2 weeks of culture.

3.3 HIV-1 isolates

HIV-$1_{Ba\text{-}L}$ was propagated and titrated in MDM. HIV-1_{IIIB} used in section 4.1.1. was grown and titrated in PBMC cultures. The TCLA strains HIV-1_{IIIB} and HIV-1_{MN} used in section 4.2.3. were continuously grown and titrated in MOLT3 and PM1 cells respectively. Primary viral isolates (HIV-1_{5508}, HIV-1_{6088} HIV-1_{10005}, HIV-1_{181}, HIV-1_{57}, HIV-1_{5233}, HIV-1_{27}, HIV-1_{26}, HIV-1_{34} and HIV-1_{130}) were isolated from PBMC of children infected by their seropositive

mothers [326]. The hematological and clinical characteristics of the patients are presented in table 3.1.

Patient	CDC stage	Total blood lymphocytes (per mm^3)	Blood $CD4^+$ cells (per mm^3)	Blood $CD8^+$ cells (per mm^3)	Serum p24 Ag (pg/ml)	Therapy
5508	A1	10530	1769	6581	0	
6088	A1	6437	3347	1609	387	
10005	B1	5589	1512	3130	500	
181	NA	NA	NA	NA	NA	NA
57	A3	2759	359	1477	500	
5233	B2	5410	909	2516	NA	
27	B3	1148	57	517	500	AZT
34	B3	3668	331	1944	250	
130	A3	2425	412	1213	300	AZT

Table 3.1 Clinical and hematological characteristics of HIV-1 infected patients *CDC stage indicates the clinical and immunological status of the child at a given age, according to the criteria of the 1994 Revised CDC Classification for Children. Categories N, A, and B denote children with no, mild, or moderate signs or symptoms of HIV-1 infection, respectively. Categories 1, 2, and 3 include children with no, moderate or severe immune suppression, respectively. AZT, zidovudine. NA, not available.*

All viruses were propagated and titrated in PBMC cultures obtained from healthy blood donors.

Virus stocks were stored at -80°C before use and treated with Rnase-free DNase I (20 U/ml; Sigma) for 30 min at room temperature before infection.

3.4 Isolation of monocytes/MDM and HIV-1 infection

PBMC were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from buffy-coat preparations obtained from healthy

donors. Cells were resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% AB^+ serum (Sigma), 20% FCS (BioWhittaker, Walkersville, MD), 2 mM glutamine, 50 μg/ml streptomycin and 100 U/ml penicillin, and cultured at a concentration of $1x10^6$ cells/cm^2 at 37 °C in 12-well tissue culture plates (Nunc, Roskilde, Denmark), in a 1 ml volume. To obtain monocytes, non-adherent cells were removed after 1 hour, and the remaining adherent cells were cultured for 24 hours. To obtain MDM, non-adherent cells were removed after 5-7 days by extensive washing with medium. Monocyte/MDM preparations contained ≥90% $CD14^+$ cells, as assessed by immunofluorescence.

For infection, MDM were incubated with viral strains ($TCID_{50}$: $50/10^6$ cells) in RPMI 1640-20% FCS, in a total volume of 0.5 ml of cell-free viral supernatant. After overnight incubation, unbound virus was removed by extensive washing, fresh medium (0.5 ml) was added, and cultures were further incubated at 37°C. Supernatants were harvested every 3 days for p24 Ag detection. Culture medium was fully replaced every 6 days, without washing.

3.5 Isolation of lymphocytes and HIV-1 infection

Normal peripheral blood lymphocytes depleted of monocytes by 2 cycles of adherence to plastic were activated by a 3 day incubation with PHA (3 μg/ml: Sigma). The resulting PHA blasts were resuspended at 2.5 x 10^6 cells/ml in medium containing 10% FCS and IL-2 (10 U/ml: Amersham, Buckinghamshire, UK), and incubated overnight with HIV-1 isolates. Subsequently, free virus was removed by washing twice with RPMI 1640, and cells ($1x10^6$/ml) were cultured in 12-well plates in the presence of IL-2. Culture

supernatants were harvested every 3-4 days, and tested for the presence of p24 Ag by ELISA.

3.6 HIV-1 detection

HIV-1 p24 Ag concentrations in culture supernatants were determined by ELISA [252]. Briefly, p24 Ag from a detergent lysate of virions was captured by an immobilized anti-p24 Ag polyclonal antibody (D7320: Aalto Bio Reagents, Dublin, Ireland). Bound p24 Ag was then detected using an alkaline phosphatase-conjugated anti-p24 Ag monoclonal antibody (BC 1071-AP: Aalto Bio Reagents) and the AMPAK ELISA amplification system (DAKO A/S. Glostrup, Denmark).

RT activity in the supernatants of HIV-infected MDM was assayed as described in Ref. [392]. Briefly, 10 μl of cell-free culture supernatants were added to 50 μl of a mixture containing poly (A), oligo (dT) (Pharmacia), $MgCl_2$, and ^{32}P-labeled deoxythymidine 5'-triphosphate (dTTP) (Amersham) in a 96-well V-bottom microtiter plate, and incubated for 1.5 h at 37°C. Five ml of the RT reaction mixture were then dotted onto DE81 paper (Whatman, Maidstone, England), dried, washed and subsequently counted on a microplate scintillation counter (Packard Instrument Co., Meriden, CT).

3.7 Preparation of LPS-conditioned and monokine-depleted supernatants

LPS-conditioned supernatants were prepared by incubating cultures of normal uninfected MDM in the presence or absence of LPS (1 μg/ml). Two days later, supernatants were harvested, centrifuged, and stored at -20 °C until used. In

order to deplete LPS-conditioned supernatants of chemokines (MIP-1α, MIP-1β, RANTES), Petri dishes were coated for 2 h at room temperature with neutralizing antibodies in PBS, at concentrations (10-30 μg/ml) expected to neutralize the amounts of chemokines found in culture supernatants. Control plates were coated with normal goat IgG (55 μg/ml). LPS-conditioned supernatants were incubated in the sensitized dishes overnight at 37°C, then collected and used immediately. Crude supernatants from MDM unstimulated or stimulated with LPS (1 μg/ml) were depleted of IFN-α and IFN-β as follows: a mixture of sheep polyclonal anti-human IFN-α and IFN-β antibody (at the concentration required to neutralize 1000 U/ml of human IFN-α/β) or sheep control IgG (50 μg/ml) were incubated with protein G-Sepharose (Pharmacia Biotech) for 1 h at room temperature. LPS-conditioned or control supernatants were then incubated with the protein G-Sepharose/antibody complex overnight at 4°C, collected, centrifuged and used immediately.

3.8 Immunofluorescence

Expression of CD14, CD4, CXCR4 and CCR5 on monocytes and MDM was assessed by indirect immunofluorescence using mAb P9, Leu 3A, 12G5, 2D7, respectively, or isotype controls.

For indirect immunofluorescence, 0.5 x 10^6 cells were incubated in 100 μl of staining buffer (RPMI 1640-10% AB^+ serum, with 0.01% sodium azide) containing purified mAbs for 30 min at 4°C, washed and further incubated with FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. Cells were then extensively washed and fixed in 4% *p*-formaldehyde. Percentages of positive cells and mean fluorescence intensity (MFI) were analyzed by a FACScan

(Becton Dickinson) gating on the monocyte population, as defined by forward and side light scatter.

3.9 Chemotaxis Assay

Cell migration was assayed in 48-well Transwell ^{TM} chambers using a 5-μm pore size polycarbonate filter membrane (Costar, Cambridge, MA). Chemokines were diluted in RPMI 1640 medium containing human serum albumin (0.3%) and added to the lower chamber. One hundred μl of a $3x10^6$/ml cell suspension were added to the upper chamber. After a 2 hour incubation at 37°C in 5% CO_2, the filter was removed and the cells migrated in the lower chamber were counted using a FACScan (Becton Dickinson) at 60 μl/min for 30 seconds. Appropriate gating on the forward and side scatter was used to select specific cell types. The chemotactic index represents the ratio between the number of cells that migrated in the presence of chemokines and those that migrate spontaneously.

3.10 DNA and RNA extraction for competitive PCR amplification

High molecular weight DNA from HIV-1 infected MDM cultures was extracted by overnight incubation at 37°C in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0, 0.3 mg/ml proteinase K) followed by extraction with a phenol-chloroform-isoamyl alcohol mixture (25:24:1) and ethanol precipitation. Total RNA was extracted according to the guanidine thiocyanate procedure [61], and treated with RNase-free DNase I (Boehringer, Mannheim, Germany) to remove traces of contaminating DNA. First-strand cDNA synthesis was obtained by priming with random hexamers and reverse transcription in 20 μl of reverse transcription mix containing 75 mM KCl, 50

mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, dNTP (Pharmacia: 0.4 mM each), MMLV-RT (Promega, Madison, WI: 400 units), RNasin (Promega: 20 units). RNA was pre-heated at 65°C for 5 min and incubated with the reaction mix at 37 °C. After 1 hour, the reaction was stopped by incubation at 95 °C for 5 min and samples were cooled on ice.

3.11 Competitive PCR amplification for CCR5 mRNA

Amplification of CCR5 cDNA was performed using primers CKR-9 (5'CATCATCCTCCTGACAATCG) and CKR-10 (5'ATGGTGAAGATAAGCCTCACAG). Quantification of CCR5 mRNA levels in MDM was carried out by a competitive PCR procedure [71] using a competitor DNA fragment carrying the primer recognition sites for β-actin (BA1 and BA4) and CCR-5 (primers CKR-9 and CKR-10). A schematic representation of this competitor is shown in Figure 4.6, panel A. and its construction is described in the legend to Figure 4.6.

Competitive PCR amplifications were carried out by adding to the sample increasing concentrations of the competitive templates, in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$) containing the two primers (100 pmol each), the four dNTPs (200 mM each) and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Emeryville, CA). Samples were submitted to 50 cycles of amplification with the following cycle profiles: denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec. After amplification, 10 µl of each PCR reaction were resolved on a 8% non-denaturing polyacrylamide gel, visualized under UV light after ethidium bromide staining and photographed. Quantification of the amplification products was obtained by densitometric scanning.

3.12 Semiquantitative PCR for HIV-1 Proviral DNA

PCR was performed in PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$) containing the primers 1 and 2II (100 pmol each) that amplify a 218 bp fragment from the HIV-1 gag gene, the four dNTPs (200 mM each) and Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD: 2.5 U). Samples were submitted to 50 cycles of amplification (95°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute). PCR products were separated on a 1.8% agarose gel, transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK) and hybridized with a gag-specific ^{32}P-labelled oligonucleotide (5'AGGCGACTGGTGAGTACGCCAAAA). In order to normalize for the quantity of DNA in each sample, a 441 bp region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified using primers 5'-GGGAAGGTGAAGGTCGGAGTC and 5'-GCTGATGATCTTGAGGCTGTTGTC. Results are expressed as the ratio between the intensities of the HIV-1 and GAPDH bands, as assessed by scanning densitometry. Each sample was amplified in duplicate or triplicate.

4 Results

4.1. Analysis of the mechanisms underlying the LPS-induced inhibition of HIV-1 replication in human macrophages

Monocyte/macrophages are critically involved in the immune response to bacterial infections. LPS/endotoxin, the major constituent of the cell wall in gram-negative bacteria stimulates monocytes/macrophages to produce cytokines and increase expression of cell adhesion molecules. LPS induces cellular responses by forming a complex with circulating LPS-binding protein and subsequently binding to CD14 [163, 340, 394]. This in turn facilitates the interaction of LPS with signaling molecules belonging to the Toll-like receptor family [179, 197, 295, 301]. LPS/CD14 interactions have been shown to result in the induction of HIV expression in monocytoid tumor cell lines [15, 297], but to protect primary macrophages from productive infection with HIV-1 *in vitro* [28, 205]. Notably, the concentrations of LPS that affect HIV-1 replication *in vitro* can be easily reached *in vivo*, and may thus affect viral replication in HIV patients superinfected with bacteria. When this work started, the mechanisms underlying the complex effects of LPS on HIV-1 expression in monocytic cells had not been elucidated. We chose to study the effects of LPS on HIV-1 expression in cultures of MDM and T cells isolated from normal donors, and infected *in vitro* with different strains of HIV-1.

4.1.1. LPS suppresses HIV-1 replication in macrophage cultures infected *in vitro*

In order to characterize the effects of LPS on the replication of HIV-1 in monocytic cells, MDM from normal donors were infected *in vitro* with the M-tropic HIV-1 $_{Ba\text{-}L}$ strain, in the presence or absence of LPS (1 μg/ml). Figure 4.1. A shows that p24 Ag secretion in untreated MDM cultures rapidly reached high levels, that were maintained for over 10 days. In contrast, p24 Ag secretion by

LPS-treated MDM remained extremely low throughout the culture time. RT activity in the same cultures showed a similar pattern (data not shown). Figure 4.1. B shows that LPS-dependent inhibition of p24 Ag secretion was also observed in MDM cultures infected *in vitro* with HIV-1_{5508}, a primary NSI isolate obtained from an asymptomatic HIV-1-infected patient.

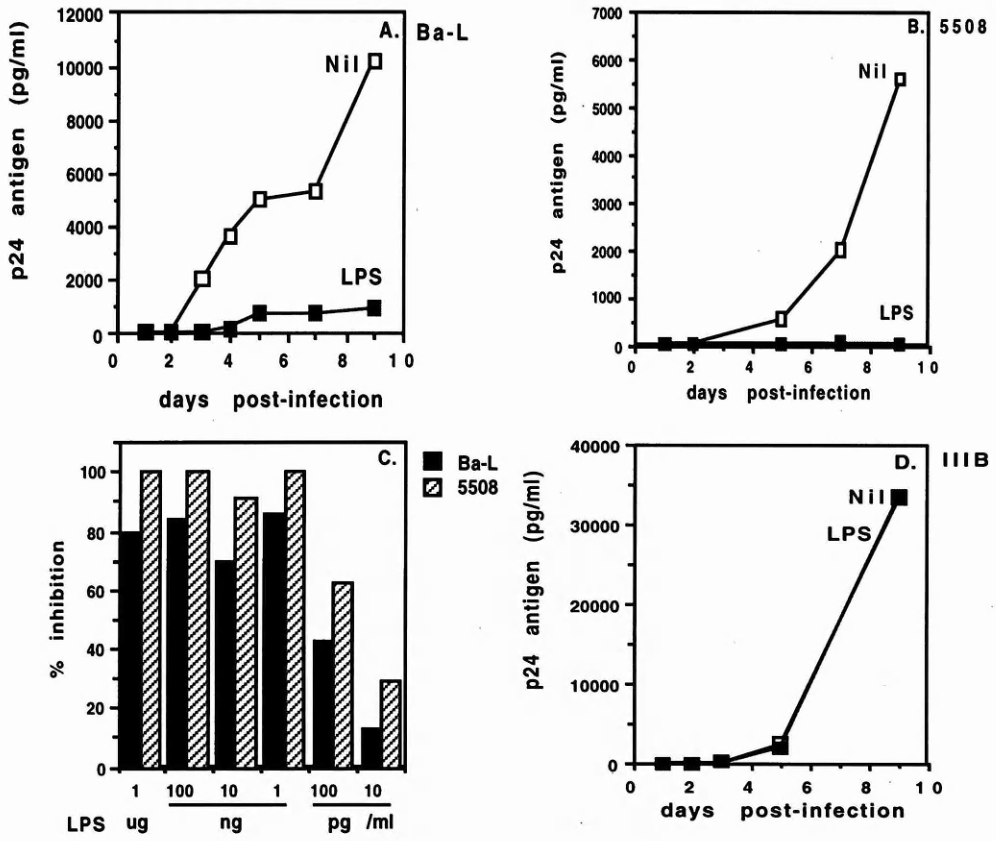

Figure 4.1 *LPS suppresses HIV-1 replication in MDM cultures infected in vitro.* MDM from healthy donors were infected with HIV-$1_{Ba\text{-}L}$ (panel A), the primary NSI isolate HIV-1_{5088} (panel B), or HIV-1_{IIIB} (panel D), all at 500 pg/ml, in the presence or absence of LPS (1 μg/ml). MDM were washed one day later and further cultured, adding LPS every 3 days. Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data are representative of 10 (panel A), 3 (panel B) and 2 (panel D) independent experiments. In panel C, MDM were infected with HIV-$1_{Ba\text{-}L}$ or HIV-1_{5508} in the presence of decreasing concentrations of LPS. p24 Ag secretion was assessed 5 days after infection.

Figure 4.1.C shows that p24 Ag secretion was inhibited by > 70% using LPS at a concentration of 1 ng/ml. Notably, inhibition was still apparent when LPS was added at 10 pg/ml, a physiologically significant concentration [297]. Interestingly, LPS addition did not inhibit HIV-1 expression in MDM cultures

infected with the SI laboratory strain, HIV-1_{IIIB} (Figure 4.1.D). The surprisingly high levels of replication of our HIV-1_{IIIB} in MDM are likely to result from multiple passages of the viral stock in human primary PBMC. Addition of LPS did not result in significant cell death, nor in apoptosis, as assessed by trypan blue or propidium iodide staining (data not shown).

LPS-induced inhibition of HIV-1 replication was dependent on the time of addition of LPS to the culture. Figure 4.2 shows that HIV-1 expression was completely blocked when LPS was added at the time of infection or 1 day later, but was affected less and less when LPS was added 2 or 3 days after infection with HIV-1. Notably, viral replication was completely inhibited by pre-treating MDM with LPS for 48 h before infection. However, the inhibitory effect of LPS pre-treatment was abolished if the cells were washed before virus addition (data not shown). These data suggest that LPS interferes with early events in HIV-1 infection.

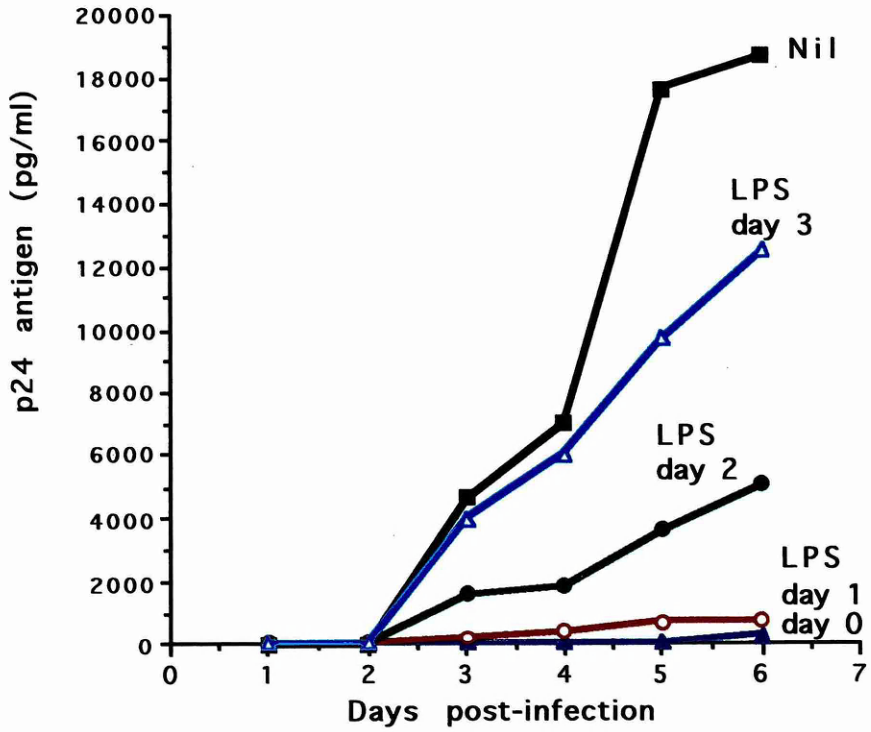

Figure 4.2 *LPS-induced inhibition of HIV-1 expression in MDM cultures is dependent on the time of addition of LPS.* MDM were infected with HIV-1 Ba-L (500 pg/ml), and stimulated with LPS (1 μg/ml) at different times from the initiation of the culture. Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data represent the mean of 2 independent experiments.

4.1.2. CD14 expression is upregulated in LPS-treated, HIV-1-infected macrophages

LPS has been shown to upregulate the expression of its own receptor, CD14, in whole blood [235]. We therefore asked whether a modulation of CD14 expression may contribute to the effects of LPS on HIV-1 replication in MDM. Immunofluorescence analysis of MDM cultures 2 days after HIV-1 infection showed that CD14 expression was upregulated not only in uninfected, LPS-treated MDM, but also in *in vitro* HIV-1-infected, LPS-untreated cells (Figure 4.3). Interestingly, LPS and HIV-1 synergized in upregulating CD14 expression. These data suggest that the combined effects that HIV-1 infection and LPS stimulation have on CD14 expression may amplify the LPS-induced, CD14-mediated suppression of HIV-1 replication.

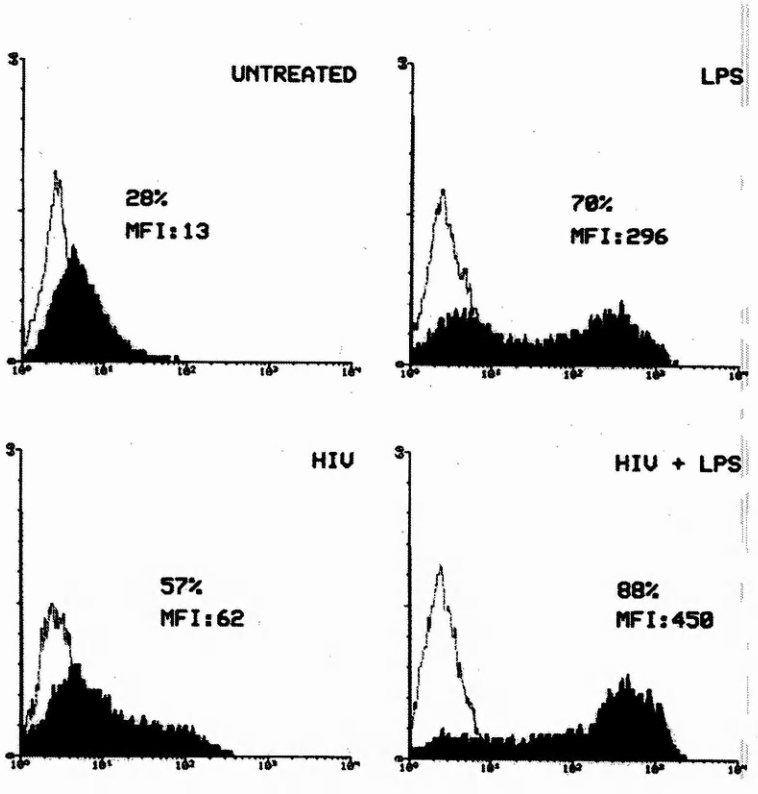

Figure 4.3 *LPS and HIV-1 synergize in upregulating CD14 expression in MDM.* MDM were infected with HIV-1_{Ba-L} (500 pg/ml) in the presence or absence of LPS (1 μg/ml). After 2 days of culture, CD14 expression was assessed by direct immunofluorescence, using phycoerythrin-conjugated mAb P9 and an unrelated isotype control. The data are representative of 3 independent experiments.

4.1.3. LPS-induced HIV-1 suppression is not mediated by an effect on the secretion of IL-6 and TNF-α

A number of cytokines have been described to regulate HIV-1 expression. In particular, TNF-α and IL-6 enhance HIV-1 replication in acutely infected MDM. The HIV-1-inducing effect of TNF-α is mainly, if not exclusively, mediated by the activation of NF-κB, which activates LTR-driven viral RNA transcription [84]. IL-6 induces expression of viral proteins and RT activity to levels comparable to those induced by TNF-α, but unlike TNF-α, does not increase significantly the levels of steady-state viral mRNA [292]. We therefore investigated whether a decrease in the production of these HIV-1 stimulatory cytokines may underlie LPS-dependent inhibition of HIV-1 replication in MDM. Figure 4.4 shows that LPS-induced IL-6 secretion was vigorous and comparable in both uninfected and HIV-1-infected MDM cultures. In contrast, infected cultures treated with LPS showed an impairment in their ability to sustain TNF-α secretion over time. However, stimulation with LPS released high and comparable levels of TNF-α (>40 ng/ml) from uninfected and infected cells at the initiation of the culture, prior to removal of unbound virus. The decrease in TNF-α detected after 2 or more days of culture did not result from masking by shed soluble TNF receptors, nor from a selective upregulation of membrane TNF-α (data not shown). Addition of rTNF-α (10 and 100 U/ml) did not restore HIV-1 expression, as detected by p24 Ag (data not shown). Thus, the decrease in TNF-α was not responsible for the inhibitory effect of LPS on HIV-1 replication. Loss of sensitivity of HIV-1-infected MDM to TNF-α-mediated upregulation of HIV expression, rather than decreased levels of TNF-α, may be involved in LPS-induced inhibition of HIV infection. The mechanisms involved in TNF-α suppression remain to established.

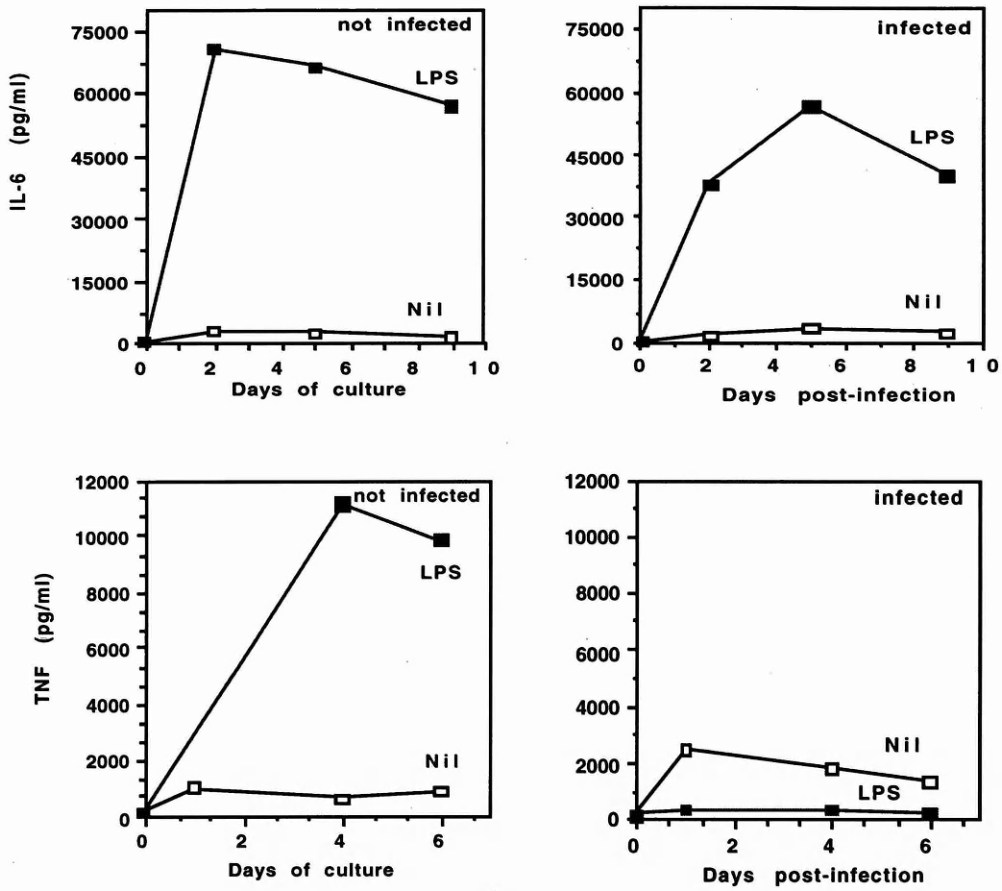

Figure 4.4 *Effects of LPS stimulation and/or HIV-1 infection on IL-6 and TNF-α secretion by MDM.* Uninfected or HIV-1_{Ba-L}-infected MDM were cultured in the presence or absence of LPS (1 μg/ml). LPS was added to the cultures every 3 days. IL-6 and TNF-α concentrations in the supernatants were measured by ELISA. The data are representative of 4 independent experiments.

4.1.4. LPS-induced inhibition of HIV-1 replication is mediated by soluble factors active on both macrophages and T lymphocytes

The finding that pre-treatment with LPS inhibited HIV-1 infection only if the cells were not washed before adding the virus prompted us to investigate whether the effects of LPS are mediated by soluble factors. To this purpose, LPS-conditioned supernatants were obtained from MDM cultures stimulated with LPS for 24 h, and LPS was neutralized by the addition of polymyxin B (15

μg/ml). Normal MDM were then infected with HIV-1 and cultured either with LPS, or with these supernatants (100% v/v) in the absence of LPS. Table 4.1 shows that supernatants from LPS-treated MDM inhibited HIV-1 replication as actively as LPS itself, even in the presence of polymyxin B.

Table 4.1 *LPS-induced inhibition of HIV replication in MDM is mediated by the release of soluble factors*

			HIV-1 p24 Ag release (pg/ml)	
Culture	**Supernatant added**	**Polymyxin**	Day 4	Day 7
MDM+HIV-1	Nil	-	3,209	12,616
MDM+HIV-1	Nil	+	3,620	13,917
MDM+HIV-1+LPS	Nil	-	108	992
MDM+HIV-1+LPS	Nil	+	2,953	12,408
MDM+HIV-1	Untreated MØ	+	3,048	13,726
MDM+HIV-1	LPS-treated MØ	+	100	300

MDM from healthy donors were infected *in vitro* with HIV-$1_{Ba\text{-}L}$, in the presence or absence of LPS (1 μg/m), LPS-conditioned supernatants (100% v/v) or polymyxin B sulfate (15 μg/ml). Supernatants from infected cultures were harvested at different time points, and assayed by ELISA for p24 Ag secretion.

Interestingly, the effect of the soluble inhibitory factor(s) was not MDM-specific. Table 4.2 shows that the same LPS-conditioned supernatants also suppressed viral expression in T lymphocytes infected with two NSI strains, HIV-$1_{Ba\text{-}L}$ and the primary isolate HIV-1_{181}. The inhibitory effect of LPS-conditioned MDM supernatants on HIV replication in T cells was particularly remarkable, because LPS *per se* had no effect when added directly to purified, infected T cells. However, LPS-conditioned supernatants failed to suppress the replication of an SI primary isolate, HIV-1_{5233}, in T cells. These results suggest

that suppressive monokines released by MDM upon stimulation with LPS are responsible for the observed inhibition of HIV replication.

Table 4.2 *Soluble factors released by LPS-treated MDM inhibit the replication of NSI HIV-1 strains in T lymphocytes*

			HIV-1 p24 Ag release (pg/ml)		
Culture	**Supernatant added**	**Polymyxin**	Ba-L	181	5233
T cells+HIV-1	-	-	2,155	8,755	7,057
T cells+HIV-1	-	+	2,355	6,390	7,592
T cells+HIV-1+LPS	-	-	2,344	7,795	6,793
T cells+HIV-1+LPS	-	+	2,086	7,885	7,738
T cells+HIV-1	Untreated MØ	+	2,225	8,927	7,462
T cells+HIV-1	LPS-treated MØ	+	< 100	281	7,198

PHA activated T lymphocytes isolated from healthy donors were infected *in vitro* with 2 NSI HIV-1 strains, HIV-1_{Ba-L} and HIV-1_{181}, or with an SI strain, HIV-1_{5233}, in the presence or absence of LPS (1 μg/ml), LPS-conditioned supernatants (100% v/v) or polymyxin B sulfate (15 μg/ml). Supernatants from infected cultures were harvested 5 days after infection, and assayed by ELISA for p24 Ag secretion.

4.1.5. C-C chemokines released by LPS-stimulated macrophages mediate the suppression of HIV-1 replication

Several monokines have been reported to suppress HIV-1 replication. Among them, IL-10 blocks HIV replication by inhibiting the secretion of endogenous TNF-α and IL-6 [386], cytokines that upregulate HIV expression. IL-1Ra, on the other hand, is produced by HIV-infected MDM in large excess relative to IL-1α and –β, and thus effectively counteracts IL-1-mediated induction of HIV expression [407]. We tested whether the release of these monokines was responsible for the LPS-induced inhibition of HIV-1 expression in MDM. To this purpose, neutralizing anti-IL-10 or anti-IL-1Ra antibodies were added to MDM cultures infected with HIV-1 and stimulated with LPS. Figure 4.5 shows that addition of neither antibody reversed the suppression of HIV-1 replication caused by LPS, thus ruling out a role of IL-10 and IL-1Ra in HIV-1 suppression.

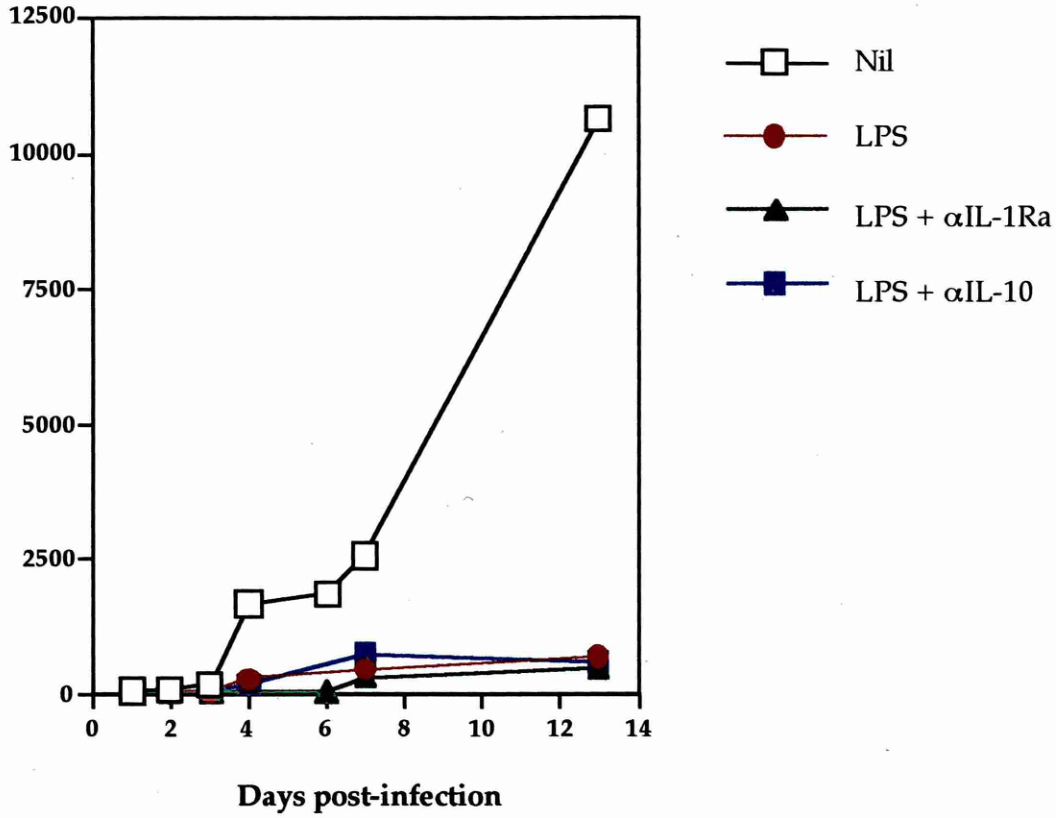

Figure 4.5 *Effects of neutralizing antibodies against HIV-1-inhibitory cytokines.* MDM were infected with HIV-1_{Ba-L} and stimulated with LPS (1 μg/ml), in presence or absence of neutralizing anti-IL-1Ra or anti-IL-10 antibodies (10 μg/ml). Culture supernatants were harvested daily, and tested for p24 Ag secretion by ELISA. The data represent the mean of 2 separate experiments. Control antibodies had no effect on p24 Ag secretion.

$CD8^+$ T lymphocytes release soluble factors that inhibit HIV-1 replication in $CD4^+$ T cells in a manner not restricted by the major histocompatibility complex [380]. HIV-1 inhibition was recently shown to largely depend on the presence of the C-C chemokines RANTES, MIP-1α and MIP-1β [67], the natural ligands of CCR5, the coreceptor for primary NSI strains [5, 93, 100]. In preliminary experiments, we therefore assessed whether CCR5 is expressed in MDM, and whether stimulation with LPS induces the release of these chemokines. Because no specific antibody was available at the time, competitive PCR experiments were carried out to quantitate CCR5 mRNA in total cDNA isolated from MDM. Quantification was achieved by using a DNA fragment that acts as a dual competitor for PCR amplification of both CCR5

cDNA and β-actin (as an internal standard) (Figure 4.6. A). Figure 4.6. B shows that high levels of CCR5 mRNA were expressed by MDM at the time of infection. Stimulation with LPS did not upregulate the expression of CCR5 in infected MDM (data not shown).

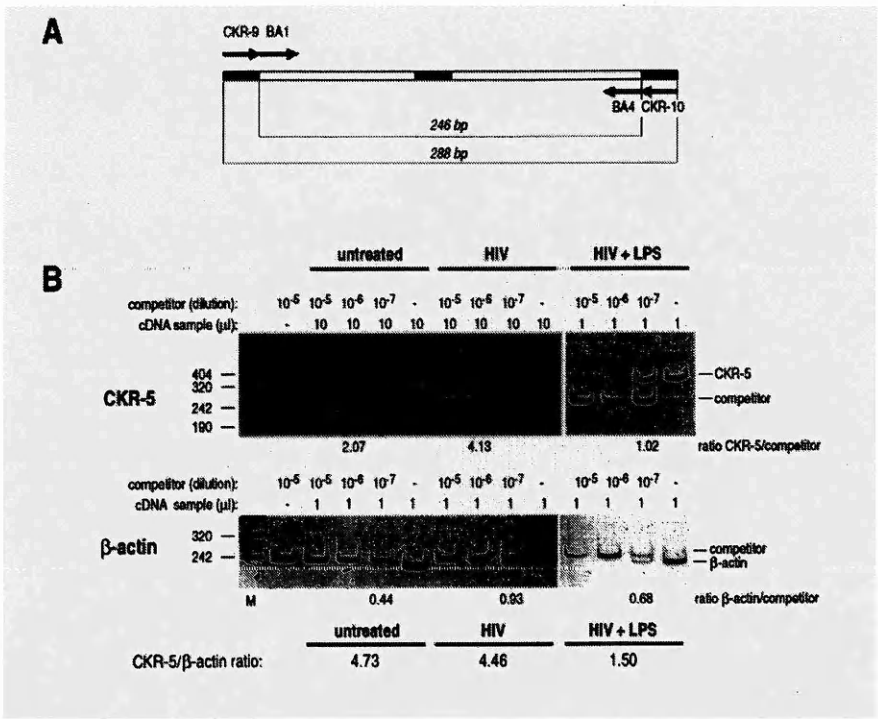

Figure 4.6 *MDM express CCR5 mRNA.* Total RNA was extracted from untreated MDM. RNA samples were treated with DNase I to remove traces of contaminating DNA and reverse transcribed using random hexameric primers. The cDNA products were mixed to scalar amounts of a synthetic competitor DNA fragment containing primer recognition sites for both β-actin and CCR5 amplification, and amplified with the respective primer pairs. (panel A) Schematic representation of the competitor DNA fragment used for the quantification of CCR5 and β-actin cDNA. The fragment contains a core sequence derived from the human β-actin cDNA, carrying a 20 bp insertion in the middle (black box). Amplification with the β-actin-specific primer set BA1-BA4 detects a 226 bp product on human cDNA, and a 246 product from the competitor DNA. To this core sequence, the primer recognition sites for human CCR5 amplification were added at the two ends (indicated by gray boxes) by re-amplification with composite primers corresponding to the CKR-9+BA1 sequence at one end and CKR-10+BA4 at the other end. Amplification with CKR-9 and CKR-10 generates a 288 bp fragment from the competitor template and a 368 bp fragment from the CCR5 cDNA. (panel B) Competitive PCR for the quantification of CCR5 and β-actin mRNAs. cDNA samples from untreated MDM were mixed with tenfold dilution of the competitor DNA fragment as indicated, and amplified with primer sets CKR-9/CKR-10 and BA1/BA4 for CCR5 and β-actin mRNA quantification. Amplification products were resolved by PAGE, stained with ethidium bromide and quantified by densitometric scanning. According to the principles of competitive PCR, quantification of the target molecules in the samples was obtained by estimation of the ratio between the amplification products, as reported at the bottom of each gel. Furthermore, since the same competitor DNA fragment acts as a competitor for quantification of both CCR5 and β-actin, standardization for mRNA input is obtained by estimating the ratio between the two measurements, as indicated at the bottom of the Figure. M: molecular weight markers.

We next investigated whether stimulation with LPS induces MDM to release C-C chemokines. Table 4.3 shows that addition of LPS resulted in vigorous production of these C-C chemokines by MDM, both uninfected and infected *in vitro* with HIV.

Table 4.3 *C-C chemokine secretion in macrophage cultures*

	day 2			day 5		
	MIP-1α	**MIP-1β**	**RANTES**	**MIP-1α**	**MIP-1β**	**RANTES**
		(pg/ml)			**(pg/ml)**	
Exp. #1						
Nil	1,710	591	198	900	610	190
LPS	39,297	17,164	12,740	2,014	2,302	2,494
HIV	2,040	627	281	1,227	1,612	542
HIV + LPS	34,452	20,253	8,380	2,266	3,719	818
Exp. #2						
Nil	380	50	38	1,200	1,300	67
LPS	22,680	37,590	12,080	25,480	47,220	2,279
HIV	1,750	2,330	47	2,960	8,200	75
HIV + LPS	21,710	20,110	8,890	7,440	13,960	332

Uninfected or HIV-1_{Ba-L} -infected MDM were cultured in the presence or absence of LPS (1 μg/ml). LPS was added to the cultures every 3 days. Supernatants were harvested after 2 and 5 days of culture. Concentrations of C-C-chemokines in the supernatants were measured by ELISA.

We then investigated whether the C-C chemokines released in LPS-conditioned supernatants played a role in the inhibition of HIV-1 replication. Simultaneous neutralization of RANTES, MIP-1α and MIP-1β has been shown to be required to abrogate the HIV suppressive effects of $CD8^+$ T cell supernatants. Thus, high concentrations of antibodies are necessary to achieve neutralization [67]. Because monocytes and MDM express all types of Fcγ receptors (CD64, CD32 and CD16), the engagement of which is known to

modulate HIV expression [367], supernatants from LPS-stimulated MDM cultures were simultaneously depleted of RANTES, MIP-1α and MIP-1β by adsorption on specific antibodies immobilized on plastic. After polymyxin B was added to neutralize LPS, chemokine-depleted supernatants were added to HIV-1-infected MDM from different donors. In the representative experiment shown in Table 4.4, LPS-conditioned supernatants completely inhibited p24 Ag secretion. Depletion of C-C chemokines neutralized the inhibitory activity of the supernatants. In contrast, supernatants adsorbed on control goat IgG were almost as inhibitory as the undepleted ones. Our data suggest that the LPS-dependent release of HIV-1 suppressive chemokines plays a major role in the inhibition of HIV-1 replication in MDM.

Table 4.4 *Antibody-mediated depletion of C-C chemokines neutralizes the HIV suppressive activity of LPS-conditioned supernatants*

				HIV-1 p24 Ag (pg/ml)
Culture	**Supernatant added**	**Polymyxin**	**Depletion**	
MDM+HIV-1	-	-	-	4,516
MDM+HIV-1	-	+	-	4,426
MDM+HIV-1+LPS	-	-	-	597
MDM+HIV-1+LPS	-	+	-	4,500
MDM+HIV-1	Untreated MØ	+	-	5,739
MDM+HIV-1	LPS-treated MØ	+	-	176
MDM+HIV-1	LPS-treated MØ	+	anti-chemokines	3,597
MDM+HIV-1	LPS-treated MØ	+	normal goat IgG	806

MDM from healthy donors were infected *in vitro* with HIV-1_{Ba-L}, in the presence of LPS-conditioned supernatants (1/3 v/v), undepleted or depleted of monokines by adsorption on specific neutralizing antibodies or control IgG immobilized on plastic. Polymyxin B, sulfate was added at a concentration of 15 μg/ml. Supernatants from infected cultures were harvested after 4 days of culture, and assayed by ELISA for p24 Ag secretion. The table shows the results of a representative experiment.

Competitive inhibition of HIV-1 co-receptor utilization by released chemokines is expected to result in the inhibition of HIV entry into MDM [5, 93, 100]. Therefore, we tested the effects of LPS and LPS-conditioned supernatants on the early stages of the HIV-1 replication cycle by assessing the levels of proviral DNA in MDM incubated with HIV-1_{Ba-L} for 14 hrs, in the presence or absence of LPS and LPS-conditioned supernatants. Proviral DNA copies were assessed by a semiquantitative nested PCR procedure, using two primer sets specific for the *pol* gene [3, 44]. The representative experiment shown in Table 4.5 demonstrates that the addition of LPS and LPS-conditioned supernatants reduced the number of viral DNA copies by 93% and 90% respectively. The finding that LPS treatment suppressed the rate of HIV-1 DNA formation at an early time after MDM infection is consistent with the reported ability of C-C chemokines to interfere with HIV-1 entry.

Table 4.5 *LPS and LPS-conditioned supernatants inhibits early step(s) in the HIV replication cycle*

Sample	n° copies	% inhibition
HIV	5,860	
LPS	407	93
LPS-conditioned supernatants	585	90

MDM from healthy donors were infected *in vitro* with HIV-1_{Ba-L} in the presence of LPS (1 μg/ml) or LPS-conditioned supernatants (100% V/V). Semiquantitative PCR for HIV-1 DNA was performed on DNA isolated 14 hrs post-infection. The table shows the number of viral DNA copies per 10^6 MDM.

4.1.6. Recombinant C-C chemokines inhibit HIV-1 replication in human macrophages

In order to assess whether C-C chemokines are sufficient to inhibit HIV-1 replication in MDM, recombinant RANTES, MIP-1α and MIP-1β were added to HIV-infected MDM, alone or in combination. Figure 4.7 (upper panel) shows that a combination of the three chemokines, each at a concentration of 50 ng/ml, inhibited the replication of HIV-$1_{Ba\text{-}L}$ in infected MDM by 76%. In the same experiments, addition of LPS reduced p24 Ag release by 75%. Among the three chemokines, RANTES was the most potent, because it inhibited HIV-$1_{Ba\text{-}L}$ infection as efficiently as LPS when used at a concentration of 250 ng/ml. Notably, the inhibitory effect of C-C chemokines on HIV-1 replication was even more pronounced in MDM cultures infected with NSI primary viral isolates. Indeed, Figure 4.7 (lower panel) shows that RANTES, MIP-1α and MIP-1β blocked the replication of HIV-1_{5508} by > 75% even when used individually at a concentration as low as 10 ng/ml. The combination of the three chemokines suppressed HIV-1_{5508} by over 90%. The concentrations of recombinant chemokines used in our experiments were physiologically significant. Indeed, assessment of the concentrations of endogenous chemokines released by MDM during the overnight incubation with virus and LPS prior to washing (data not shown) demonstrated that at the time of *in vitro* infection, HIV is exposed to similar amounts of chemokines. These results altogether show that recombinant chemokines are sufficient to inhibit HIV infection in human MDM.

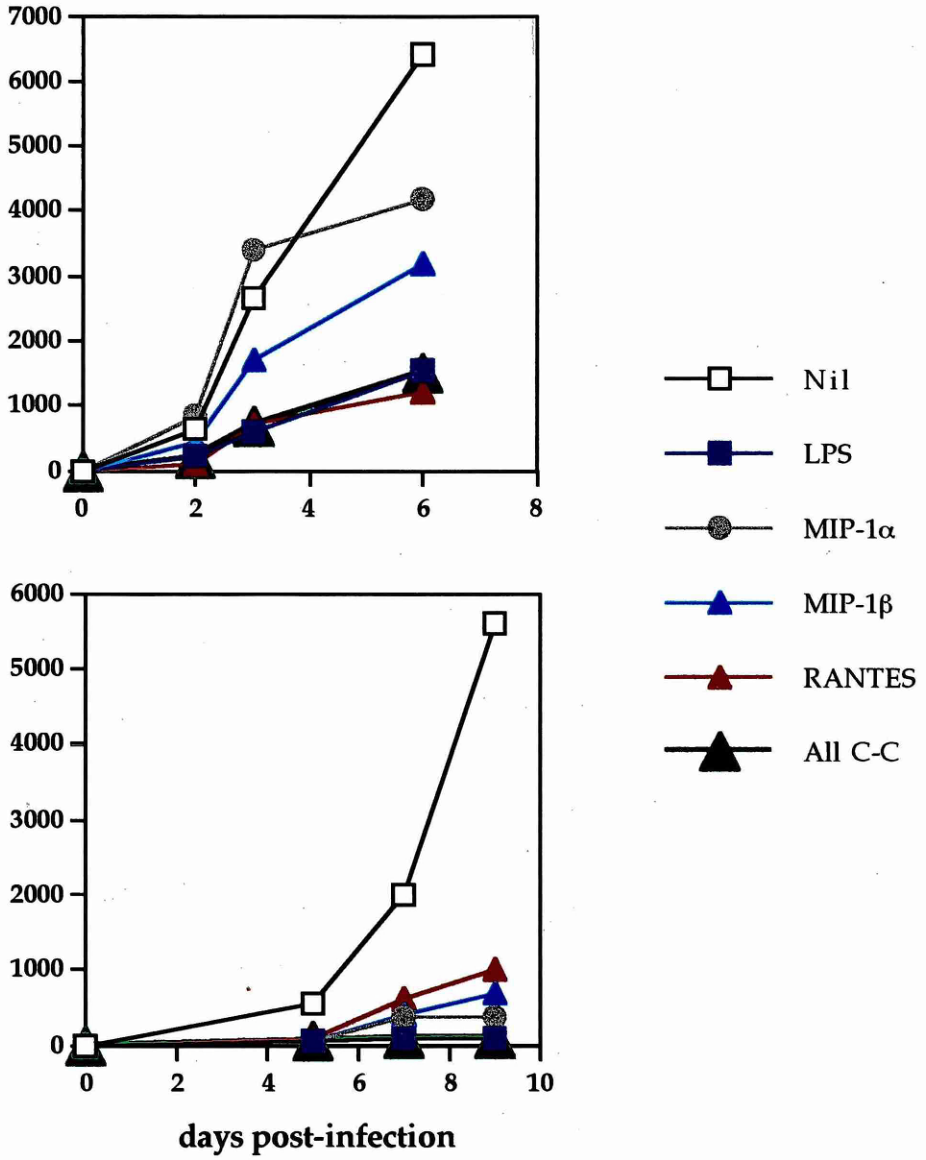

Figure 4.7 *Recombinant C-C chemokines inhibit HIV-1 replication in human MDM.* MDM from healthy donors were infected *in vitro* with HIV-1_{Ba-L} (top panel) or with the NSI primary viral isolate HIV-1_{5508} (bottom panel), in the presence or absence of LPS (1 μg/ml) and recombinant chemokines. Chemokines were added to HIV-1_{Ba-L}-infected cultures at a concentration of 250 ng/ml when used individually, and 50 ng/ml each when used in combination. For HIV-1_{5508}-infected cultures, chemokines were used at 10 ng/ml, individually and in combination. Supernatants from infected cultures were harvested at different time points, and assayed by ELISA for p24 Ag secretion.

4.2 Characterization of the role of CXCR4 in the infection of macrophages by X4 HIV-1 isolates

The identification of chemokine receptors as HIV coreceptors provided a molecular basis for the different tropism of different HIV-1 strains. In particular CCR5, the RANTES, MIP-1α and MIP-1β receptor, was shown to serve as the main coreceptor for NSI viruses [5, 60, 93, 98, 100] whereas CXCR4/fusin, the natural receptor for SDF-1 [33, 273], mediates the entry of SI HIV-1 strains, both primary and TCLA [120].

While the lack of CCR5 expression on most T cell lines [396] offered a rationale for the inability of NSI strains to infect these cells, the issue of MDM infection by HIV-1 strains with an SI phenotype remained unresolved. Although macrophages express significant levels of CXCR4 on their membranes [240], this coreceptor was assumed to be non-functional for infection [406]. Because of the critical role of macrophages in the pathogenesis of HIV-1 infection, we addressed this apparent paradox by infecting normal human MDM in vitro with a panel of primary HIV-1 isolates and TCLA strains rigorously characterized for coreceptor usage. Furthermore, we added the natural CXCR4 ligand, SDF-1, to specifically block CXCR4-mediated viral entry.

4.2.1. CXCR4 expression on human macrophages

As a first step in assessing the role of CXCR4 in MDM infection by SI HIV-1 isolates, we analyzed CXCR4 expression on monocytes and MDM from normal donors. Figure 4.8 shows that immunofluorescence with mAb 12G5 detected variable but significant levels of CXCR4 protein on monocytes one day after isolation, as assessed by both percentage of positive cells and MFI. Although culture-induced differentiation resulted in a progressive decrease of CXCR4, the receptor was expressed at comparable intensity on MDM at the time of infection (day 5 of culture, MFI: 20-29) and on MOLT-3 cells, a T cell line widely used to expand SI HIV-1 strains (MFI: 24).

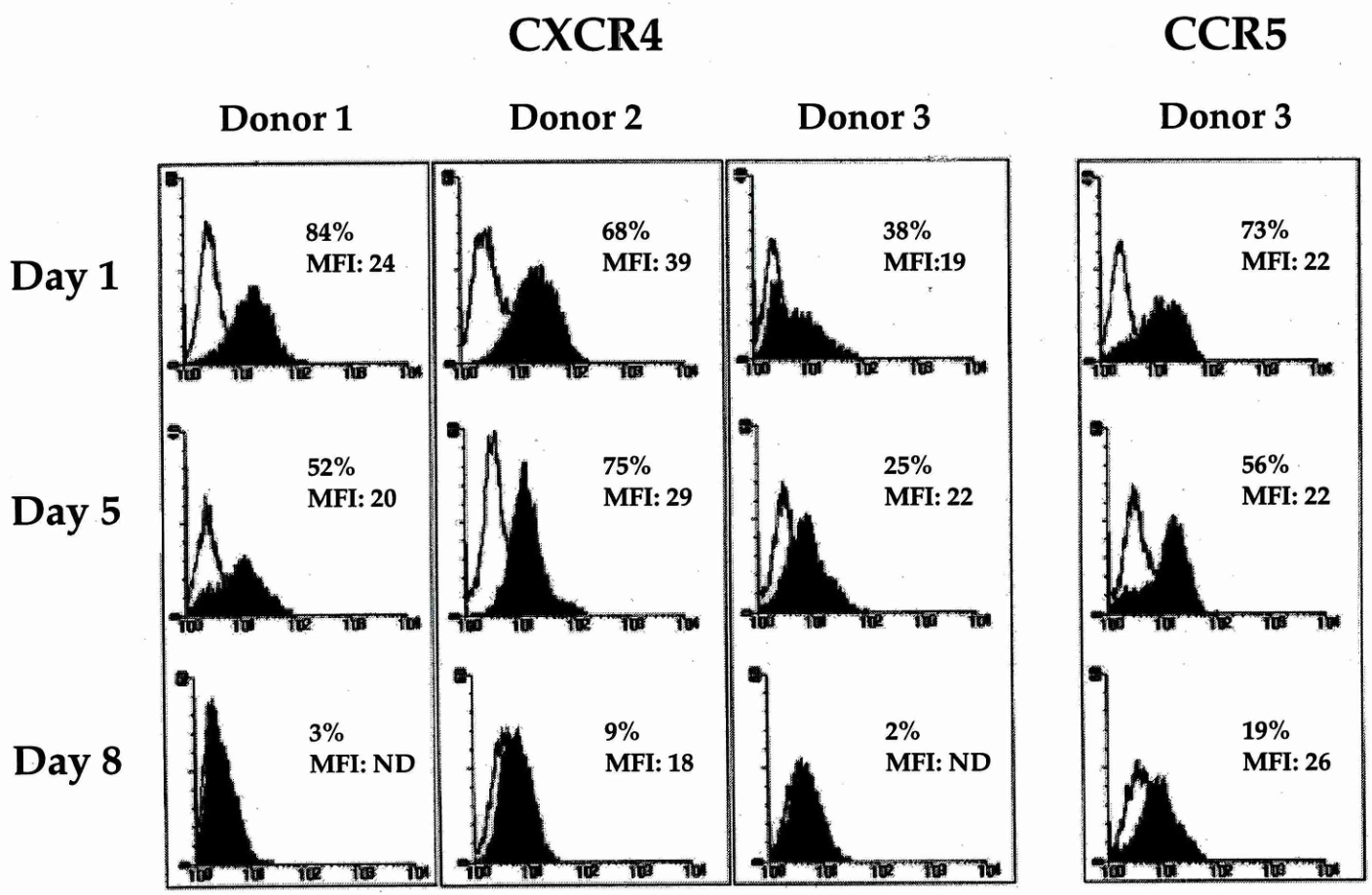

Figure 4.8 *Immunofluorescence analysis of CXCR4 and CCR5 expression in monocytes and macrophages from healthy donors.* The viral coreceptors were detected at different culture times by indirect immunofluorescence [377] using mAb 12G5 and 2D7, respectively.

The expression of the other major HIV-1 coreceptor, CCR5, followed a similar pattern in all donors examined (n=3).

4.2.2. CXCR4 is a functional chemokine receptor in human macrophages at the time of *in vitro* infection

In order to assess the functional activity of CXCR4 on MDM at the time of *in vitro* infection, we evaluated SDF-1 mediated macrophage migration and, as a control, the chemoattractant activity exerted by RANTES, a natural ligand for CCR5. At day 1 from isolation, SDF-1 induced chemotaxis of human monocytes over a wide concentration range. Notably, the levels of CXCR4 available on MDM at day 5 (the time of *in vitro* infection) were still sufficient to support a brisk chemotactic response to recombinant SDF-1 (Figure 4.9 left panel). RANTES-induced chemotaxis was in a comparable range (Figure 4.9 right panel). These results show that, at the time of virus exposure, CXCR4 is not only expressed on MDM at substantial levels, but is fully functional.

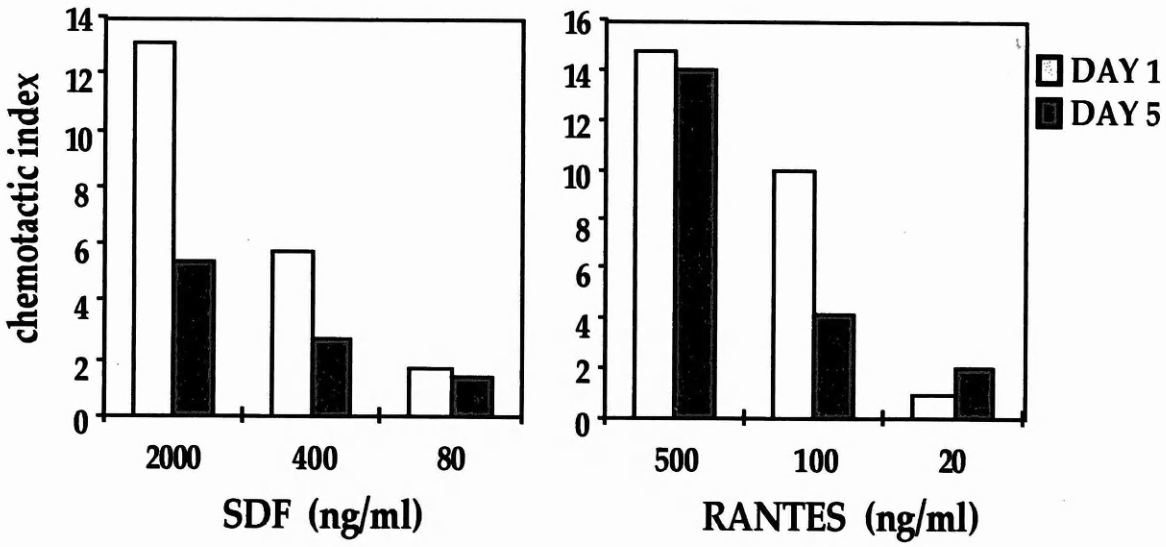

Figure 4.9 *Chemotactic activity of SDF-1 and RANTES on monocyte/ macrophages.* The chemotactic index represents the ratio between the number of cells that migrated in the presence of chemokines and spontaneous migration. The figure shows the results of 1 representative experiment out of 3.

4.2.3. Primary CXCR4-dependent HIV-1 isolates infect macrophages productively and are specifically blocked by SDF-1

In order to define the role of CXCR4 in MDM infection by HIV-1, we selected from a panel of 33 primary HIV-1 isolates rigorously characterized for coreceptor usage [326] 3 isolates that use exclusively CXCR4, together with a control group of 3 CCR5-dependent isolates. As expected, MDM were efficiently infected by primary NSI HIV-1 isolates. In a representative experiment, p24 Ag levels 9 days post-infection of cultures with HIV-1_{5508}, HIV-1_{6088} and HIV-1_{10005} were 5.6, 2.3, and 7.2 ng/ml, respectively. Notably, MDM were efficiently infected also by all the CXCR4-dependent primary SI strains, with p24 Ag release rapidly reaching substantial levels (Figure 4.10, panel A). The source of HIV in our cultures were likely to be MDM, rather than contaminating T cells. Indeed, when non-adherent CD3+/CD14- cells were infected with the same isolates 5 days after purification, no p24 Ag secretion was ever detected in spite of intense surface expression of CXCR4 (data not shown).

SDF-1 has been recently shown to be the natural ligand for CXCR4 but not for the other chemokine receptors that mediate HIV-1 fusion and entry [33, 273]. In order to formally prove that CXCR4 acts as a coreceptor for MDM infection by primary SI HIV-1 isolates, we infected MDM in the presence or absence of rSDF-1 (2 μg/ml). Figure 4.10 (panel B) shows that addition of rSDF-1 blocked the replication of all the CXCR4-dependent primary HIV-1 strains. SDF-1-dependent inhibition of MDM infection was even more efficient than that previously observed with human PBMC [326].

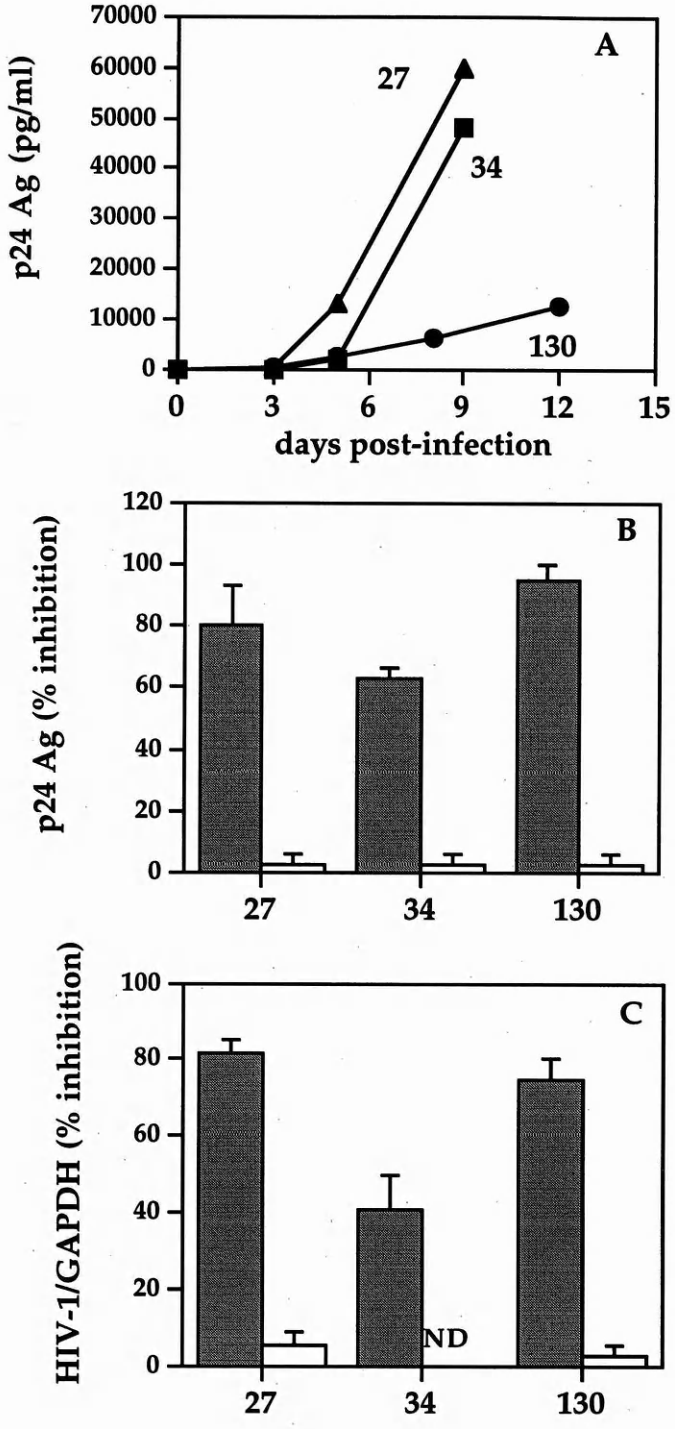

Figure 4.10 *Primary CXCR4-dependent HIV-1 isolates infect MDM and are specifically blocked by SDF-1.* **(A)** MDM from healthy donors were infected with primary CXCR4-dependent isolates (HIV-1_{27}, HIV-1_{34} and HIV-1_{130}). The Figure shows results obtained in 1 representative experiment out of 15. **(B)** SDF-1 (2 μg/ml: grey bars) and RANTES (100 ng/ml: white bars) were added at the time of infection and then every 3 days. The Figure shows the decrease (%) in p24 Ag secretion in chemokine-treated cultures at day 7 (mean of 3 experiments). **(C)** The effect of SDF-1 (grey bars) and RANTES (white bars) on viral entry was assessed by semiquantitative PCR. The Figure shows the decrease (%) in proviral DNA (HIV-1/GAPDH ratio) 14 hours post-infection (mean of 2 experiments).

Semiquantitative PCR analysis (Figure 4.10, panel C) revealed an SDF-1-induced decrease in proviral DNA 14 hours after infection that pointed to a block at the level of viral entry. By contrast, SDF-1 had no inhibitory effect on infection by CCR5-dependent HIV-1 isolates (data not shown).

Consistent with the selective use of CXCR4 as a coreceptor for entry, addition of the CCR5 ligand RANTES at a concentration (100 ng/ml) that completely inhibits infection with NSI isolates (26) did not affect the entry or the replication of CXCR4-dependent HIV-1 isolates (Figure 4.10, panels B and C). These data demonstrate that CXCR4 is a functional coreceptor for the entry of primary HIV-1 SI isolates in MDM. Further supporting this conclusion, Table 4.6 shows that MDM from a ccr5Δ32 homozygous individual could be infected by 2 HIV-1 primary isolates (HIV-1_{34} and HIV-1_{130}) that use selectively CXCR4, as well as by a primary isolate (HIV-1_{57}) that uses both CXCR4 and CCR5. Addition of SDF-1 efficiently blocked HIV infection by all viruses. By contrast, RANTES had no significant effect (data not shown).

These results altogether show that CXCR4 supports CCR5-independent HIV-1 entry in macrophages.

Table 4.6 *Primary SI HIV-1 isolates infect CCR5-null MDM and are specifically blocked by SDF-1*

HIV-1 isolates	**Coreceptors**	**SDF-1**	**p24 Ag (pg/ml)**	
			Day 5	*Day 9*
34	CXCR4	-	4,299	ND
		+	696	ND
130	CXCR4	-	1,546	7,405
		+	< 100	145
57	CXCR4, CCR5	-	792	1,889
		+	<100	<100

An individual homozygous for the ccr5Δ32 mutation was identified by RT-PCR-mediated amplification of cDNA isolated from MDM using primers previously described [377] The mutation was confirmed by direct sequencing of the PCR product. CCR5Δ32 MDM were infected with primary HIV-1 isolates, in the presence or absence of rSDF-1 (2 μg/ml). SDF-1 was added every 3 days. p24 Ag secretion in culture supernatants was determined by ELISA.

4.2.4. Infection of MDM by CXCR4-dependent TCLA HIV-1 strains

We then compared the ability of CXCR4-dependent TCLA strains and primary isolates to productively infect MDM. Proviral DNA was assessed 14 hours post-infection with 2 TCLA strains (HIV-1_{IIIB} and HIV-1_{MN} grown in MOLT3 and PM1 cells respectively) and 3 primary isolates (HIV-1_{27}, HIV-1_{34}, HIV-1_{130}). Table 4.7 shows that the level of viral entry was variable but overall comparable for TCLA strains and primary isolates. However, productive infection could not be detected with TCLA strains, even when entry had occurred with substantial efficiency (e.g., HIV-1_{IIIB} for donor 1 and HIV-1_{MN} for donor 2). These results suggest that low or absent viral replication in MDM infected with TCLA HIV-1 strains was due to both entry and post-entry defects.

Table 4.7 *Infection of MDM by CXCR4-dependent TCLA strains and primary HIV-1 isolates*

HIV-1 isolates:	**IIIB**	**MN**	**130**	**34**	**27**
Donor 1					
HIV/GAPDH	17.3	ND	11.1	ND	ND
p24 Ag	<0.1	ND	7.1	ND	ND
Donor 2					
HIV/GAPDH	0.1	0.8	0.3	0.7	0.6
p24 Ag	0.1	0.7	23.7	40.4	30.0
Donor 3					
HIV/GAPDH	<0.1	0.2	7.8	10.7	0.3
p24 Ag	<0.1	<0.1	15.8	23.3	6.9

MDM from 3 donors were infected with TCLA strains and primary HIV-1 isolates. PCR for HIV-1 proviral DNA and GAPDH was performed on DNA isolated from MDM 14 hours post-infection. The Table shows the ratio between the HIV-1 and GAPDH signals, as assessed by scanning densitometry. p24 Ag secretion (ng/ml) 14 days after infection was determined by ELISA.

4.3 LPS inhibits the replication of primary X4 HIV-1 isolates In macrophages and T lymphocytes through different mechanisms

As discussed in section 1.3.3 of the *Introduction*, various microbial pathogens and/or their products can directly affect HIV replication. Dual infection of individual cells with HIV-1 and herpes virus or human HTLV-1 [109, 339] resulted in enhanced expression of HIV. Similarly, HIV-infected cells superinfected with or exposed to products of *Mycobacterium tuberculosis* and *Toxoplasma gondii* [216] showed enhanced expression of HIV. The finding that CXCR4 is a functional coreceptor for HIV-1 infection of human macrophages prompted us to investigate the effects of bacterial LPS on macrophages infected with X4 primary isolates. The rationale for this study was provided by the notion that macrophages are the major source of HIV during opportunistic infections [275] and CXCR4-dependent viruses commonly emerge at the advanced stages of infection when bacterial superinfections can frequently occur.

4.3.1 LPS and soluble factors released by LPS-treated macrophages inhibit the replication of HIV-1 isolates in MDM irrespective of coreceptor usage

We previously showed that LPS and LPS-conditioned MDM supernatants block the replication of R5 HIV-1 isolates in MDM. Inhibition was mediated by the release of C-C chemokines, in particular RANTES, MIP-1α and MIP-1β, as shown by experiments with specific neutralizing antibodies and addition of recombinant chemokines [377] (see section 4.1). We then asked whether LPS and/or soluble factors released by MDM upon LPS stimulation affect the replication of HIV-1 isolates that use coreceptors other than CCR5 for entry into target cells. Table 4.8 shows that p24 Ag release 7 days post-infection was

strongly inhibited by addition of LPS (1 μg/ml) to cultures infected by R5 and X4 HIV-1 isolates, as well as in MDM infected by a viral isolate capable of using CCR5, CXCR4 and CCR3. This result appear to be conflicting with the lack of LPS-induced suppression of HIV-1_{IIIB} replication in MDM showed in figure 4.1 panel D. The explanation is probably related to an uncontrolled, and probably too high, concentration of virus used during the overnight infection of macrophage cultures in that experiment.

Table 4.8 *LPS and soluble factors released by LPS-treated MDM inhibit the replication of HIV-1 isolates in MDM irrespective of coreceptor usage*

				HIV-1 p24 Ag release (pg/ml)					
			HIV-1:	R5	R5/X4/R3	X4			
Cells	**LPS**	**Sup**	**PM**	#10005	#5233	#26	#27	#27*	#130
MDM	-	-	-	7,180	2,666	3,915	1,081	8,553	2,295
MDM	+	-	-	<100	306	1,007	200	1,118	330
MDM	+	-	+	7,620	1,836	ND	2,720	9,330	2,565
MDM	-	MØ	+	4,985	2,942	11,117	ND	9,695	2,615
MDM	-	LPS/MØ	+	<100	519	615	<100	252	<100

MDM from healthy donors or from an individual homozygous for the Δ32 *ccr5* mutation (*) were infected in vitro with R5 (HIV-1_{10005}), R5/X4 (HIV-1_{5233}) and X4 (HIV-1_{26}, HIV-1_{27}, HIV-1_{130}) HIV-1 primary isolates in the presence or absence of LPS (1 μg/m), LPS-conditioned supernatants (1:3, v/v) or polymyxin B sulfate (PM: 15 μg/ml). Supernatants from infected cultures were harvested at day 7, and assayed by ELISA for p24 Ag secretion. Addition of polymyxin in the absence of LPS had no effect on infection by HIV-1. ND: not determined

Inhibition by LPS was specific because it was fully neutralized by polymyxin B sulfate, an antibiotic that binds and neutralizes LPS [236]. Interestingly, LPS-conditioned supernatants containing polymyxin B induced a comparable decrease in p24 Ag secretion irrespective of the coreceptor(s) used by the viral

isolates. These results suggest that the LPS-dependent blockade of HIV-1 replication was mediated by LPS-released soluble factors.

4.3.2 LPS inhibits the entry of X4 HIV-1 isolates in MDM

In order to determine which steps in MDM infection with X4 HIV-1 isolates are inhibited by LPS, we used a semiquantitative PCR [376] to assess levels of proviral DNA 14 hours post-infection of MDM exposed to the virus in the presence or absence of LPS (1 μg/ml). p24 Ag secretion in the same cultures was measured 7 days after infection. Table 4.9 shows that addition of LPS dramatically decreased both proviral DNA and p24 Ag levels in MDM cultures infected with all the X4 primary viral isolates tested. As expected, entry of an R5 isolate was inhibited as well.

Table 4.9 *LPS inhibits the entry of X4 HIV-1 isolates in MDM*

HIV-1 isolates:		**#6088 (R5)**	**#130 (X4)**	**#26 (X4)**	**#27 (X4)**
	LPS				
HIV/GAPDH	-	10.28	11.1	13.9	3.2
	+	3.0	0.7	1.6	0.7
p 24Ag (ng/ml)	-	7.3	1.4	3.9	6.3
	+	<0.1	0.3	1.0	0.7

MDM were infected with R5 and X4 primary HIV-1 isolates, in the presence or absence of LPS (1 μg/ml). PCR for HIV-1 proviral DNA and GAPDH was performed on DNA isolated from MDM 14 hours post-infection. The Table shows the ratio between the HIV-1 and GAPDH signals, as assessed by scanning densitometry. p24 Ag secretion (ng/ml) 7 days after infection was determined by ELISA.

These results indicate that LPS stimulation of MDM results in a block of HIV-1 entry that affects both CCR5- and CXCR4-dependent primary viral isolates.

4.3.3 IFN-α secreted by LPS-stimulated MDM inhibits the replication of X4 HIV-1 isolates

The finding of an LPS-dependent inhibition of HIV-1 entry into MDM did not rule out the possibility of a concomitant effect of LPS and/or LPS-released

mediators on later stages in the viral life cycle. Indeed, addition of LPS to MDM cultures is known to induce the release of several monokines, including IFN-α, a factor which potently inhibits HIV-1 replication by interfering with post-entry events [177, 360]. We therefore asked whether secretion of IFN-α by LPS-stimulated MDM contributed to the suppression of HIV-1 replication observed in the presence of LPS and LPS-conditioned supernatants. To this purpose we first assessed IFN-α concentrations in the supernatants of MDM cultures treated with LPS for 24 hours. In 3 independent experiments, IFN-α levels ranged between 1 and 7 ng/ml. Infection with HIV-1 did not result in a significant release of IFN-α (data not shown). Table 4.10 shows that recombinant IFN-α strongly inhibited replication of all the HIV-1 X4 isolates tested when added at 1,000 U/ml. However, suppression by more physiologic IFN-α concentrations (100 U/ml, i.e. ≈ 27 ng/ml) was less efficient and consistent. These results suggest that secretion of IFN-α is one of the mechanisms through which LPS blocks infection of macrophages with X4 HIV-1 isolates.

Table 4.10 *IFN-α blocks the replication of X4 HIV-1 isolates in macrophages*

			HIV-1 p24 Ag release (pg/ml)			
Culture	**Sup added**	**PM**	**HIV-1$_{26}$**	**HIV-1$_{27}$**	**HIV-1$_{27}$***	**HIV-1$_{34}$**
HIV-1	-	-	3,915	15,206	8,553	2,835
HIV-1+IFN-α (100 U/ml)	-	-	ND	3,545	ND	2,906
HIV-1+IFN-α (1000 U/ml)	-	-	<500	2,717	483	321
HIV-1	MØ	+	11,117	17,380	9,695	5,053
HIV-1	LPS/MØ	+	615	1,212	772	365

MDM from healthy donors or from an individual homozygous for the Δ32 *ccr5* mutation (*) were infected in vitro with X4 HIV-1 primary isolates in the presence or absence of LPS (1 μg/m), LPS-conditioned supernatants (1:3, v/v), polymyxin B sulfate (PM: 15 μg/ml) or rIFN-α (100 or 1,000 U/ml). Supernatants from infected cultures were harvested at day 7, and assayed by ELISA for p24 Ag secretion.

4.3.4 LPS, but not IFN-α, downregulates the expression of the receptor and coreceptors for HIV-1 on MDM

Because PCR analysis had provided evidence for an LPS-dependent inhibition of the entry of X4 HIV-1 isolates in MDM, we next investigated whether addition of LPS or IFN-α affected the expression of CD4 and/or CXCR4, the receptor and coreceptor for these viral isolates. To this purpose, MDM were incubated in the presence of LPS (1 μg/ml) for 24 hrs, and then assessed for CD4 and CXCR4 expression by indirect immunofluorescence. Membrane CCR5, which is expected to be downregulated by LPS [338] and/or LPS-induced CC-chemokines [6, 231], was tested in parallel. Figure 4.11 shows the results obtained in one representative experiment. LPS treatment induced a dramatic downregulation of CD4 expression in MDM and a marked decrease in CXCR4. CCR5 was barely detectable on the surface of LPS-treated MDM, and CD14 was strongly enhanced, as previously reported by our group (data not shown) [377]. In contrast, figure 4.12 shows that addition of IFN-α had no inhibitory effect on CD4 and CXCR4 levels, despite the high cytokine concentration used (1,000 U/ml). Notably, CCR5 expression was unaffected as well.

These results suggest that downregulation of both CD4 and CXCR4 may be an important mechanism underlying the LPS-dependent block in the entry of HIV-1 X4 isolates in MDM.

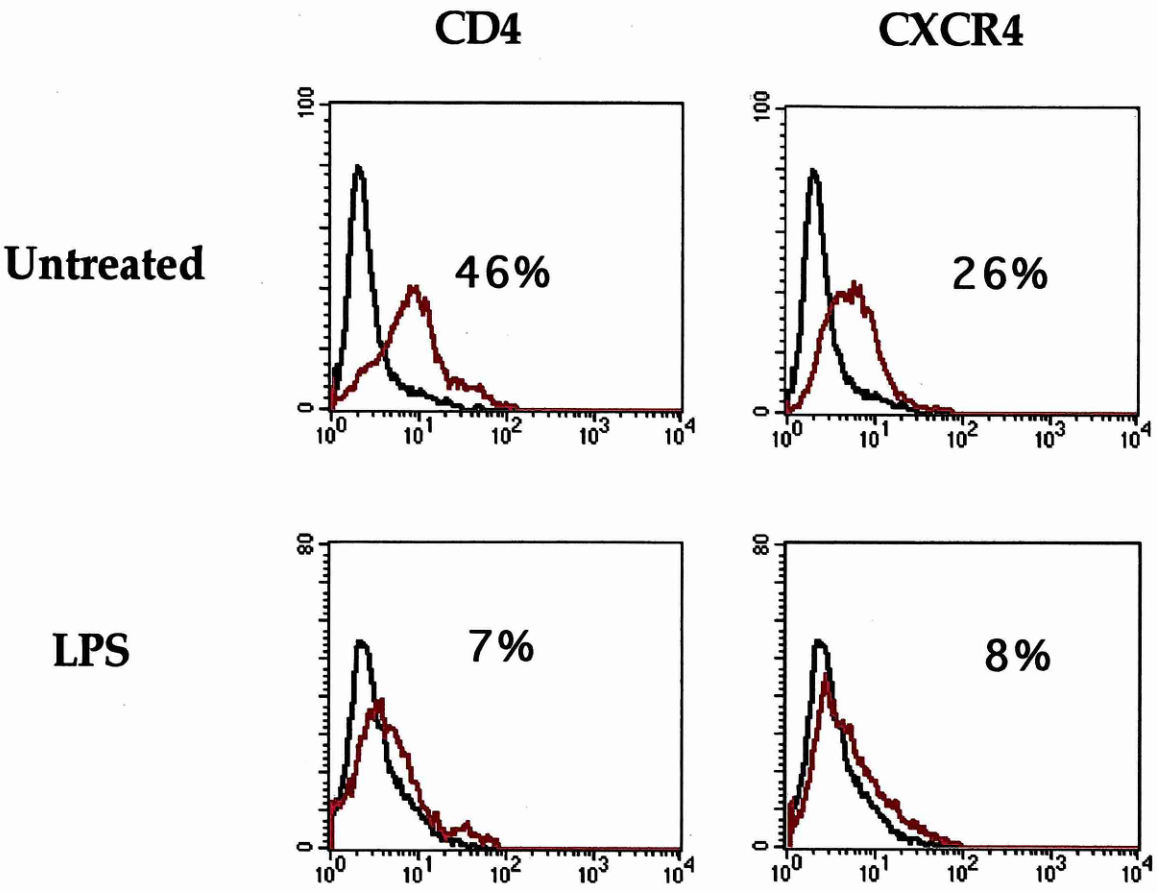

Figure 4.11 *Immunofluorescence analysis of CD4 and CXCR4 expression in macrophages LPS-stimulated.* MDM were cultured in the presence or absence of LPS (1 μg/ml) for 24 h. The viral receptor and coreceptor were detected by indirect immunofluorescence using mAb Leu 3A and 12G5, respectively, and an unrelated isotype control. The data are representative of 3 independent experiments.

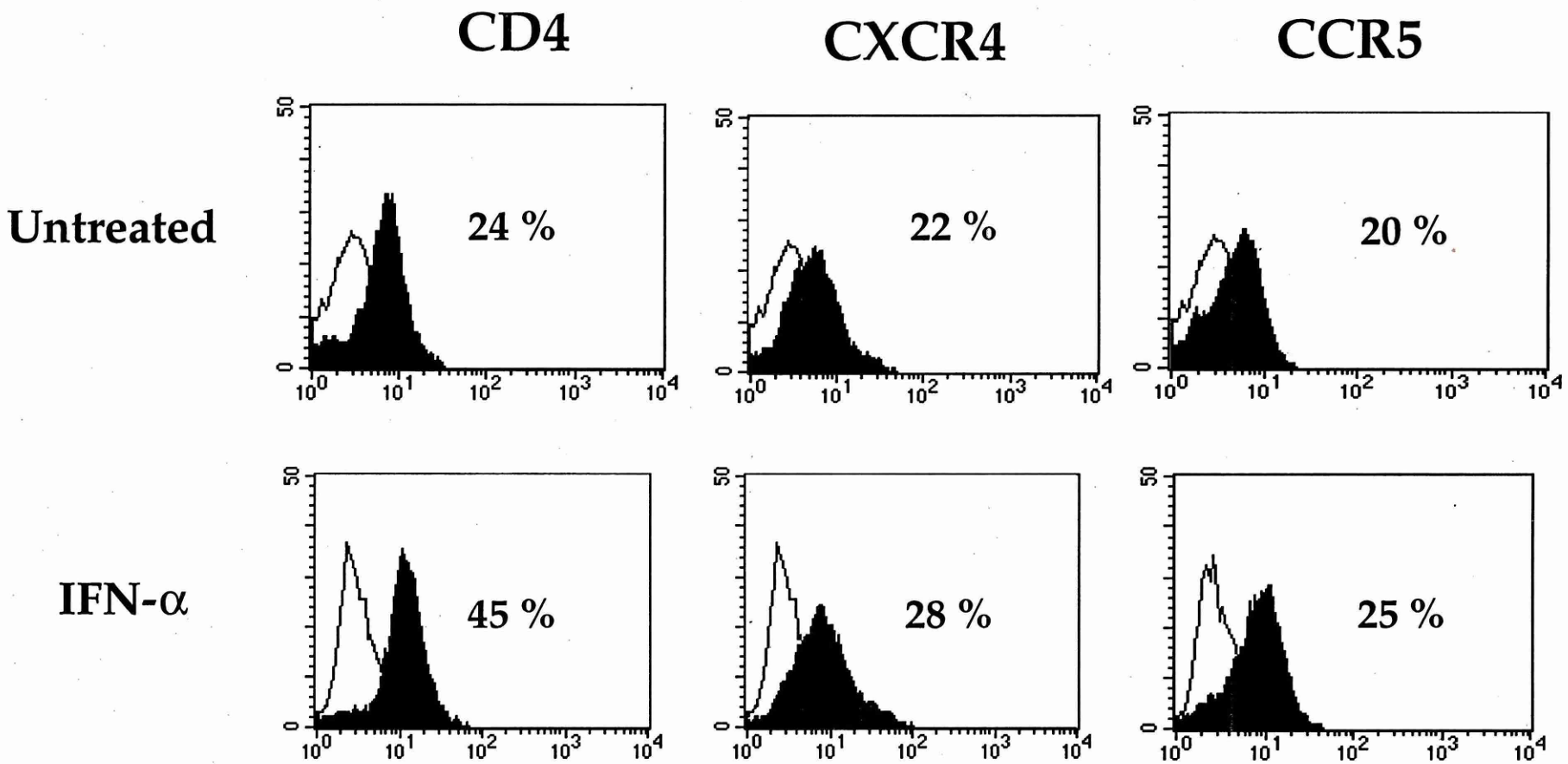

Figure 4.12 *IFN-α does not affect the expression of HIV-1 receptors in macrophages* MDM were cultured in the presence or absence of IFN-α (1000 U) for 24 h. The viral receptor and coreceptor were detected by indirect immunofluorescence using mAb Leu 3A, 12G5, 2D7 respectively and an unrelated isotype control. The data are representative of 3 independent experiments.

4.3.5 IFN-α and LPS-conditioned supernatants, but not LPS, block infection of T-lymphocytes by X4 HIV-1 isolates

Human T lymphocytes, the major target of HIV-1 infection *in vivo* together with macrophages, are known to be LPS-unresponsive. However, these cells closely interact with macrophages and are thus exposed to their secretory products, including the ones induced by LPS during bacterial infections. Indeed, we showed that C-C chemokines released by LPS-stimulated MDM suppressed infection with R5 isolates in both macrophages and T cells [377]. We therefore asked whether infection of T lymphocytes with X4 primary isolates could also be affected by the addition of LPS-conditioned supernatants. In parallel, we tested the effects of recombinant IFN-α under the same experimental conditions. Table 4.11 shows that both soluble macrophage-derived factors and IFN-α strongly suppressed infection of T cells with all the X4 isolates used. By contrast, LPS *per se* had no inhibitory effect.

Table 4.11 *rIFN-α and soluble factors released by LPS-treated MDM inhibit the replication of X4 HIV-1 isolates in T lymphocytes*

			HIV-1 p24 Ag release (pg/ml)		
Culture	**Sup added**	**Polymyxin**	**HIV-1_{27}**	**HIV-1_{34}**	**HIV-1_{26}**
PBL+HIV-1	-	-	29,358	22,733	31,200
PBL+HIV-1+LPS	-	-	25,858	24,000	29,016
PBL+HIV-1+IFN-α	-	-	4,922	7,546	2,209
PBL+HIV-1	MØ	+	30,041	29,233	19,276
PBL+HIV-1	LPS/MØ	+	4,478	2,720	3,454

PBL from healthy donors were infected in vitro with X4 primary HIV-1 isolates in the presence or absence of LPS-conditioned supernatants (1:3 v/v, with polymyxin B sulfate, 15 μg/ml) and rIFN-α (1,000 U/ml). Supernatants from infected cultures were harvested at day 7, and assayed by ELISA for p24 Ag secretion.

4.3.6 Expression of CD4 and CXCR4 on T lymphocytes is not affected by LPS-conditioned supernatants or IFN-α

In order to investigate the mechanisms through which IFN-α and LPS-released factors suppress the replication of CXCR4-dependent HIV-1 isolates in T-lymphocytes, we then tested whether these stimuli could affect the expression of CD4 and CXCR4. CCR5 was also assessed, as an internal control. Figure 4.13 shows the results obtained in one representative experiment out of three. Immunofluorescence analysis revealed that expression of CD4, CXCR4 and CCR5 was readily detectable in unstimulated cells. IFN-α had no effect on the surface levels of the molecules under investigation. Addition of LPS-conditioned supernatants, on the other hand, left CD4 and CXCR4 expression unaffected, but suppressed CCR5, probably through downmodulation of the receptor by LPS-released C-C chemokine ligands [377]. Surface expression of CD4, CCR5 and CXCR4 was not modulated by treatment with LPS or polymyxin B alone (data not shown). These results show that inhibition of the replication of X4 isolates in lymphocytes upon incubation with LPS-conditioned supernatants cannot be ascribed to a downregulation of HIV-1 receptors and coreceptors.

4.3.7 Suppressive factor(s) released by LPS-stimulated MDM but not IFN-α block HIV-1 entry in T lymphocytes

The finding that the expression of CD4 and CXCR4 in T cells was not downmodulated upon treatment with LPS-conditioned supernatants suggested that different mechanism(s) may be involved in the LPS-dependent inhibition of HIV-1 infection in monocytes and T cells. Therefore, we tested whether HIV-1 entry in T lymphocytes was affected by LPS-derived soluble suppressive factors. To this purpose, we assessed levels of proviral DNA in T lymphocyte cultures incubated with primary X4 isolates for 14 h in the presence or absence of LPS-conditioned supernatants or IFN-α.

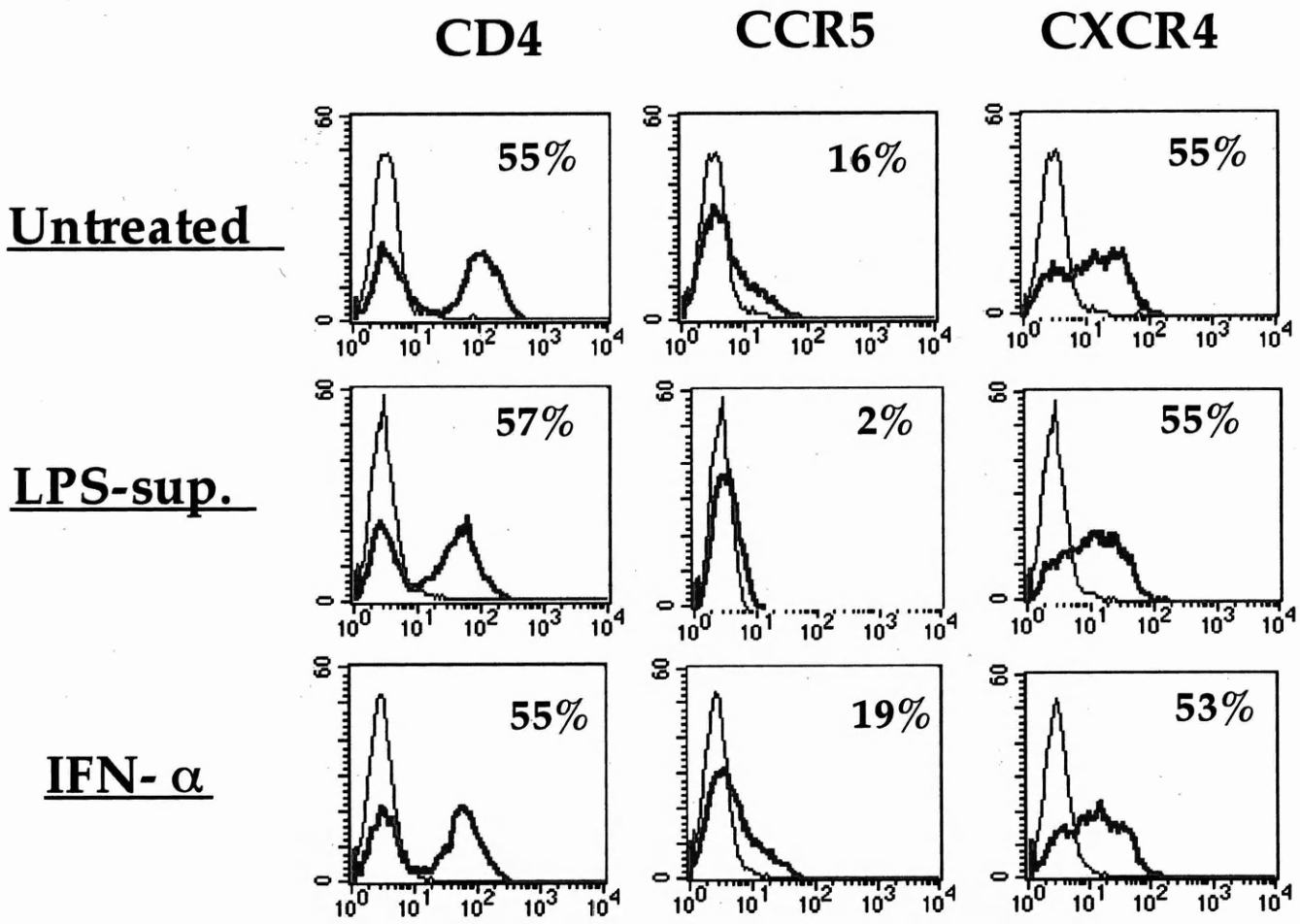

Figure 4.13 *Effects of LPS-conditioned supernatants and IFN-α on the expression of CD4, CXCR4, and CCR5 in human T lymphocytes.* PBL from healthy donors were stimulated with LPS-conditioned supernatants (1:3 v/v, with polymyxin B sulfate, 15 μg/ml) or IFN-α (1000 U). After 1 day of culture, CD4, CXCR4, and CCR5 expression was assessed by indirect immunofluorescence, using mAb Leu 3A, 12G5 and 2D7, respectively. The data are representative of 3 separate experiments.

Table 4.12 shows that LPS-conditioned supernatants, but not IFN-α, strongly decreased the levels of HIV-1 proviral DNA 14 h post-infection. By contrast, both stimuli inhibited p24 Ag secretion at 10 day from infection.

Table 4.12 *Entry of CXCR4-dependent isolates into primary T lymphocytes is inhibited by LPS-conditioned supernatants*

	Nil	LPS-supernatants	IFN-α
HIV/GAPDH	43,97	5,04	39,35
p24 Ag	8,735	1,200	1,314

PHA activated T lymphocytes from a healthy donor were infected with primary X4 HIV-1 isolates. PCR for HIV-1 DNA and GAPDH was performed on DNA isolated 14 hours post-infection. The Table shows the ratio between the HIV-1 and GAPDH signals, as assessed by scanning densitometry. p24 Ag secretion (ng/ml) 10 days after infection was determined by ELISA.

To definitively prove that IFN-α is not sufficient to mediate the complex HIV suppressive activity of LPS, supernatants from macrophages stimulated with LPS were depleted of both type I interferons (IFN-α and IFN-β) using specific antibodies, and then added to PHA activated T lymphocytes infected with X4 primary isolates, in the presence of polymyxin B. Table 4.13 shows that LPS-conditioned supernatants completely inhibited HIV-1 replication. Antibody-mediated depletion of tipe I IFNs only modestly neutralized the inhibitory activity of LPS-conditioned supernatants, and was not significantly more effective than depletion mediated by control IgG. The neutralizing activity of the antibodies against IFN-α has been tested in a separate experiment (data not shown).

These results overall indicate that the release of IFN-α/β may contribute to the LPS-induced containment of HIV-1 replication. However, LPS-treated

macrophages appear to secrete additional soluble factor(s) that, unlike IFN-α/β, markedly suppress the entry of X4 isolates in both macrophages and T cells.

Table 4.13 *Depletion of IFN-α and IFN-β does not neutralize the HIV suppressive activity of LPS-conditioned supernatants*

				HIV-1 p24 Ag (pg/ml)
Culture	**Supernatant added**	**Polymyxin**	**Depletion**	
HIV-1	-	-	-	73,600
HIV-1+LPS	-	-	-	65,850
HIV-1+LPS	-	+	-	98,800
HIV-1+ IFN-α	-	-	-	10,080
HIV-1	Untreated MØ	+	-	81,400
HIV-1	Untreated MØ	+	anti-IFN-α/β	65,625
HIV-1	LPS-treated MØ	+	-	1,111
HIV-1	LPS-treated MØ	+	anti-IFN-α/β	7,902
HIV-1	LPS-treated MØ	+	normal goat IgG	2,020

PHA activated T lymphocytes from healthy donors were infected *in vitro* with the X4 primary isolates HIV-1_{26}, in the presence of LPS-conditioned or unstimulated supernatants (1/3 v/v), undepleted or depleted of IFN-α/β. Polymyxin B, sulfate was added at a concentration of 15 μg/ml. p24 Ag secretion (ng/ml) 5 days after infection was determined by ELISA.

5.DISCUSSION

5.1 LPS inhibits infection by R5 HIV-1 isolates in human macrophages

For several years it has been known that stimulation with bacterial LPS protects macrophages from productive infection by HIV-1 *in vitro* [28, 205]. Despite the potential implications of this finding for the pathogenesis and treatment of HIV infection, the mechanisms responsible for the HIV suppressive effect of LPS remained unknown. Our results show that LPS stimulates human MDM to release soluble factors - the C-C chemokines RANTES, MIP-1α and MIP-1β - that strongly inhibit HIV replication, not only in macrophages but also in T lymphocytes. Furthermore, our data suggest that CCR5, the receptor for RANTES, MIP-1α and MIP-1β, is the major cofactor for the entry of R5/NSI isolates in human macrophages, as well as T cells. This hypothesis is supported by a number of findings presented herein: (1) at the time of infection, MDM expressed CCR5 at the level of both mRNA and surface protein; (2) stimulation with LPS induced the release of endogenous C-C chemokines, and reduced viral DNA load in infected MDM by > 90% as early as 14 hrs after infection, suggesting an inhibition at the level of viral entry; (3) depletion of C-C chemokines strongly reduced the HIV-suppressive capacity of LPS-conditioned MDM supernatants; (4) recombinant chemokines at physiologically significant concentrations inhibited the replication of HIV-1 NSI strains in MDM.

The suppressive effect of C-C chemokines on MDM infection by R5 HIV-1 strains was also supported by the observation that recombinant RANTES, MIP-1α and MIP-1β potently inhibited fusion between primary macrophages and HIV-1_{Ba-L} Env-expressing cells [5]. However, the issue of C-C chemokine-

induced inhibition of HIV-1 replication in macrophages was controversial, with some groups supporting this possibility [11, 51, 377, 405] and others reporting that entry of NSI HIV-1 strains into primary macrophages was relatively insensitive to C-C chemokines[100, 254, 274, 328, 341]. This discrepancy was likely to be caused by differences in experimental conditions, such as methods chosen for MDM propagation and stimulation and/or virus source and/or readout of the experiments and/or time of addition of chemokines relative to virus. Several lines of evidence have linked the antiviral activity of β chemokines with surface expression of heparan sulfate proteoglycans (HSPG). Indeed, chemokines are basic proteins that bind avidly to negatively charged proteoglycans and this interaction appears to be essential for their HIV suppressive effect [274]. In addition, it has been recently reported that RANTES, MIP-1α, and MIP-1β are secreted by CTL as a macromolecular complex containing sulphated proteoglycans [379]. These findings suggest that the association between chemokines and HSPGs expressed on the cell surface and/or secreted may contribute to the specific binding of RANTES, MIP-1α, and MIP-1β to CCR5, allowing its downmodulation and thus facilitating a marked HIV-1 inhibition. The different effects of β chemokines in macrophages versus T cells may be explained by the observation that lymphocytes constitutively express cell surface HSPGs. By contrast, proteoglycan expression in macrophages is a tightly regulated process that depends on the state of cellular differentiation and activation [370]. Indeed, high concentrations of RANTES had no antiviral activity when MDM were generated using exogenous growth factors such as macrophage colony-stimulating factor (M-CSF) or granulocyte/macrophage (GM)-CSF, whereas lower concentrations strongly inhibited infection of MDM obtained by 5-day adherence. Notably,

macrophages prepared following these two protocols express different amounts of surface proteoglycans, and chondroitin sulfate removal partially abolished the antiviral effect of RANTES on MDM cultured for 5 days without other stimuli [8].

The observation that RANTES, MIP-1α, and MIP-1β suppress HIV-1 at the level of virus entry provided a rationale for the findings that LPS potently stimulates HIV-1 replication in the latently infected U1 cells, and induces LTR-directed transcription in transfected monocytoid tumor cell lines [15, 297]. Indeed, LPS is known to potently activate the cellular transcription factor NF-κB that, in turn, stimulates LTR-driven gene expression or virus production by cells uniformly infected in a latent state.

While the results obtained with recombinant chemokines clearly show that these chemoattractants are sufficient to suppress R5 isolates replication in MDM, it is possible that LPS-conditioned supernatants contain additional factor(s) with HIV suppressive effects. For example, consistent with previous reports [145], we found that IFN-α is released by LPS-stimulated MDM and blocks the replication of both R5 and X4 isolates. LPS may affect HIV infection of MDM by yet another mechanism, i.e., through a direct and unusually sustained downregulation of surface CCR5 expression that results from altered recycling of chemokine receptors [128].

5.2 CXCR4 is a functional coreceptor for HIV-1 infection of primary macrophages

The availability of assays that determine HIV-1 coreceptor usage, and of ligands that selectively block HIV entry, provided a rational way out of the pre-existing maze of viral phenotypes and nomenclatures, and prompted us to readdress

the issue of macrophage infectability by primary HIV-1 strains with different biological properties. Section 4.2 of *Results* shows that human MDM can be efficiently infected by primary HIV-1 isolates that selectively use CXCR4 as a coreceptor. This notion is supported by a rigorous characterization of all the relevant viral isolates as selective CXCR4 users, by the demonstration that CXCR4 is functional in an independent assay (i.e., chemotaxis), and most importantly, by the ability of SDF-1, the natural ligand of CXCR4, to prevent HIV-1 infection. We conclude that MDM support the entry and replication not only of R5, but also of X4 primary HIV-1 isolates. Our conclusion is consistent with analogous results showing that several X4 primary isolates replicated equally well in macrophages with or without CCR5, and were inhibited by different ligands for CXCR4, including SDF-1 and the bicyclam derivative AMD3100 [343, 403, 404]. Moreover, AMD3100 and SDF-1 were able to block infection of Δ32/Δ32 CCR5 macrophages by SI strains that used a broad range of coreceptors including CCR3, CCR5, CCR8, CXCR4, and BONZO [343]. These overall observations strongly imply that, together with CCR5, CXCR4 is the predominant coreceptor used for HIV-1 infection.

The ability of HIV to efficiently use CXCR4 to infect macrophages has clinical implications. In addition to their well established role in the early stages of the disease and in viral transmission [245], macrophages are also a source of virus during the opportunistic infections that mark the progression of HIV-1 disease [275] and a target for the CXCR4-dependent HIV-1 strains that emerge in the late stages of HIV infection [326].

Although CXCR4 was initially cloned by a cDNA library derived from primary human macrophages, some studies documented the resistance of macrophages to infection by several X4 strains, thus providing an explanation

for the selective transmission of M-tropic strains during mucosal exposure to virus [406]. As for the "chemokine inhibition dilemma", these discrepancies have been ascribed to variations in macrophage isolation and culture methods [312], temporal modulation of CXCR4 levels during cell culture [264], varying sensitivities of different assay systems for coreceptor function or HIV-1 infection, and, most importantly, to the different biological properties of primary X4 isolates and prototype X4 laboratory strains (such as HIV-1_{IIIB} and HIV-1_{NL4-3}) passaged innumerable times since their initial isolation.

Therefore our findings, together with analogous results from other groups, cast some doubt on the traditional definition of HIV-1 tropism based on infection of cells manipulated by culture conditions, and more generally, on the usefulness of thinking about HIV isolates as M- versus T-tropic. In particular, the conflicting results obtained with CXCR4-dependent TCLA HIV-1 strains underline how the cellular tropism of HIV isolates appears to be determined by multiple virus/host cell interactions. Blocks have been observed at the entry step, and have been ascribed to limited coreceptor availability [404] and/or to intrinsic defects in the fusogenic properties of env proteins [45, 213]. Post-entry defects have also been described, implicating the cellular factors required to activate viral replication [126, 183, 327, 342]. In this respect, the transcription factors NF-ATc [195] and GATA-3 [402] activate HIV-1 transcription and replication in T cells, whereas binding of C/EBP proteins to the HIV-1 LTR is required for HIV-1 replication in MDM [166]. By the same token, the HIV-1-encoded protein *vpr* is important for efficient viral replication in primary MDM, but not activated T cells [73]. It is tempting to speculate that HIV-1 strains continuously grown in T cell lines might become highly dependent on T cell-specific transcription factors for their replication,

and/or might develop mutations in genomic regions critical for replication in macrophages. Such events would remain functionally silent as long as the virus is passaged in T cells, but would be likely to undermine replication in macrophages.

5.3 LPS inhibits the infection of X4 isolates in human macrophages

Because we were able to show that CXCR4 is a functional coreceptor for HIV-1 not only in T cells but also in macrophages, the evaluation of the role played by LPS during infection with X4 isolates became critical for our analysis of monocyte/HIV-1 interactions. Interestingly, as already observed for R5 viruses, we found a strong reduction in the replication of X4 HIV-1 isolates following LPS stimulation of human macrophages. Furthermore, soluble suppressive factor(s) released upon LPS treatment were able to neutralize infection with CXCR4-dependent viruses in macrophages as well as T lymphocytes. Infection of both cell types appeared to be blocked mainly at the level of entry. HIV suppression in macrophages was shown to result in part from the ability of this bacterial component to downregulate expression of both CXCR4 and CD4. By contrast, surface expression of these receptors was not affected in T lymphocytes, suggesting a different mechanism of action. Inhibition of HIV-1 entry was unrelated to the release of IFN-α/β. Indeed, unlike LPS-conditioned supernatants, IFN-α did not reduce proviral DNA levels at early times post-infection; moreover, depletion of IFN-α/β from LPS-conditioned supernatants did not neutralize their inhibitory potential. The observation that supernatants from LPS-treated macrophages inhibit cell entry of X4 isolates, together with the finding that macrophages did not secrete SDF-1 (the only known natural ligand for CXCR4) strongly points to the existence of additional soluble

suppressive factor(s) so far uncharacterized. The nature of these potential HIV-suppressive molecule(s) is currently under investigation.

In this regard, some papers reported enhanced secretion of macrophage-derived chemokine (MDC) upon LPS stimulation [311]. This β chemokine was identified based on its ability to block replication of both R5 and X4 HIV isolates in PBMCs [277] and therefore was a suitable candidate to mediate the effects of LPS. Nevertheless, the inhibitory activity of MDC is still controversial, because it has been detected by one group using synthetic MDC, but not by others (us included) using recombinant forms of the protein [217]. The mechanism through which MDC would inhibit both R5 and X4 isolates also remains elusive. Indeed, the only known receptor for MDC is CCR4 [186], a molecule that does not appear to have HIV coreceptor activity. Last but not least, unlike LPS-derived factors, MDC has been reported to affect post-entry steps in the HIV cycle. Thus it is unlikely that MDC release accounts for the LPS-dependent inhibition of HIV infection of T cells and macrophages observed in our experiments, since cell entry appears to be affected in both cell types.

The finding that LPS-stimulated macrophages release soluble factors that effectively inhibit HIV replication in both macrophages and T cells may prompt a reinterpretation of the role played by bacterial superinfections in the pathogenesis and progression of HIV infection. It has been recently shown that CD14 is not just the receptor for LPS of gram negative bacteria [394], but is a pattern recognition receptor for foreign lipoglycans of gram positive bacteria and mycobacteria as well [208, 300]. Thus, a vast array of exogenous stimuli derived from microbial pathogens may conceivably trigger intense chemokine release. In this perspective, the effect of bacterial superinfections in patients with HIV-1 immunodeficiency may be complex and somewhat

counterintuitive. The chemokine response triggered by the infectious agent upon interaction with the host's macrophages may in fact contribute to the containment of HIV-1 infection in the main targets of the virus, T cells and mononuclear phagocytes.

Altogether, the data presented in this thesis may help redefine our current understanding of the role played by monocyte/macrophages in the pathogenesis of HIV infection. Macrophages have been viewed mostly negatively, as major targets for HIV [143, 384], reservoirs for the virus [201, 351], triggers for T cell apoptosis [122, 170, 397], and as a source of soluble factors (TNF-α, IL-1, IL-6) that sustain viral replication [125, 292, 294]. The potential for a defensive role of macrophages only became clear after macrophages-derived C-C chemokines were shown to exert a potent inhibitory effect on HIV replication [67]. These chemoattractants are vigorously secreted not only by $CD8^+$ T lymphocytes, the cells traditionally implicated in HIV-1 suppression, but also by activated monocyte/macrophages [259]. Therefore, our results raise the possibility that macrophages may play a dual role in HIV infection.

6. References

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